Laboratory Procedure Manual

Analyte: **Phytoestrogens : Daidzein, Enterodiol, Enterolactone, Equol, Geinstein, O-Desmethylangolensin**

Matrix: **Urine**

Method: **HPLC-ESI-MS/MS**

Method No: 4069.03

Revised: March 2018

as performed by: Nutritional Biomarkers Branch (NBB)
Division of Laboratory Sciences (DLS)
National Center for Environmental Health (NCEH)

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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

This document details the Lab Protocol for testing the items listed in the following table.

This method file describes measurements of U1PHYTO_H_R and U2PHYTO_H_R. One method was used to measure both the 24 hour urine phytoestrogen, 1st urine collection and 24 hour urine phytoestrogen, 2nd urine collection. However, these results are released as 2 separate data files.

File Name	Variable Name	SAS Label (and SI units)
U1PT_H_R U2PT_H_R	UR1DAZ	Daidzein, Urine 1st collection (ng/mL)
	UR1DMA	o-Desmethylangolensin, Urine 1st Collection (ng/mL)
	UR1EQU	Equol, Urine 1st Collection (ng/mL)
	UR1ETD	Enterodiol, Urine 1st Collection (ng/mL)
	UR1ETL	Enterolactone, Urine 1st Collection (ng/mL)
	UR1GNS	Genistein, Urine 1st Collection (ng/mL)
	UR2DAZ	Daidzein, Urine 2nd collection (ng/mL)
	UR2DMA	o-Desmethylangolensin, Urine 2nd Collection (ng/mL)
	UR2EQU	Equol, Urine 2nd Collection (ng/mL)
	UR2ETD	Enterodiol, Urine 2nd Collection (ng/mL)
	UR2ETL	Enterolactone, Urine 2nd Collection (ng/mL)
	UR2GNS	Genistein, Urine 2nd Collection (ng/mL)

1. Summary of Clinical Relevance and Principle

A. Clinical Relevance

Phytoestrogens are plant-derived polyphenolic compounds, such as isoflavones, lignans, coumestans and stilbenes that bear structural similarities to endogenous estrogens and are capable of estrogen-receptor binding **[1-4]**. Their endocrine activity, as well as their potential influence on other biologic pathways, has led to considerable interest in phytoestrogens from an epidemiological standpoint **[5]**. The consumption of diets high in phytoestrogen-rich foods has been associated with lower rates of such hormone-dependent cancers as breast **[1,2]** and prostate **[3,4]** cancer, with modulation of osteoporosis **[6]**, with reduced severity of menopausal symptoms **[7,8]**, and with lower risk for cardiovascular disease **[9,10]**. Whether phytoestrogens are indeed the active components responsible for these benefits, however, has come under scrutiny **[5,11]**, and the significance of their purported health benefits has been challenged **[12]**. Individual studies and meta analyses have often resulted in apparently conflicting findings, such as whether phytoestrogens do **[13]** or do not **[14]** significantly reduce the frequency and intensity of menopausal hot flushes. Potential toxic effects associated with phytoestrogen exposure have also been identified **[11]**. Although phytoestrogens are not acutely toxic in large dose animal tests, they have caused reduced reproductive capability in animals at chronic dietary doses; some studies suggest adverse effects on the immune system. After ingestion, the natural conjugated phytoestrogens are hydrolyzed to their aglycones (free form), absorbed, and glucuronidated in the intestine. The major circulating forms of the isoflavones are the glucuronidated species **[12]**; glucuronidated forms also predominate in the urine **[13]**.

B. Test Principle

The test principle utilizes high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) for the quantitative detection of genistein, daidzein, equol, O-desmethylangolensin, enterodiol, and enterolactone. Human urine samples are processed using enzymatic deconjugation of the glucuronidated phytoestrogens followed by size-exclusion filtration. Phytoestrogens are then separated from other urine components by reversed phase HPLC, detected by ESI-MS/MS, and quantified by isotope dilution. Assay precision is improved by incorporating carbon-13 labeled internal standards for each of the analytes, as well as a 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate, and carbon-13 labeled 4-methylumbelliferone to monitor deconjugation efficiency. This selective method allows for rapid detection of six phytoestrogens in human urine with limits of detection in the low parts per billion (ppb; ng/mL) range.

2. Safety Precautions

Consider all urine specimens as potentially positive for infectious agents including HIV, hepatitis B and hepatitis C. We recommend the hepatitis B vaccination series for all analysts working with urine. Observe universal precautions; wear protective gloves, lab coat, and safety glasses during all steps of this method. Discard any residual sample material by autoclaving after analysis is completed. Place all disposable plastic, glassware, and paper (pipet tips, sample preparation plates, gloves etc.) that contact urine in a biohazard autoclave bag and keep these bags in appropriate containers until sealed and autoclaved. Use disposable bench diapers during sample preparation and urine handling and discard after use. Also, wipe down all contaminated work surfaces with a 10% bleach solution when work is finished.

Handle acids and bases (which are used for preparation of ammonium acetate buffers) with extreme care; they are caustic and toxic. Handle organic solvents only in a well-ventilated area or, as required, under a chemical fume hood.

Reagents and solvents used in this study include those listed in Section 6. Safety data sheets (SDSs) for all chemicals are readily available in the SDS section as hard copies in the laboratory. SDSs for other chemicals can be viewed at http://intranet.cdc.gov/ossam/workplace-safety/safety-practices/chemical-safety/index.html.

3. Computerization; Data System Management

During sample preparation and analysis, samples are identified by their sample ID. The sample ID is a number that is unique to each sample that links the laboratory information to demographic data recorded by those who collected the sample.

The raw data file and respective batch file from the tandem mass spectrometer are collected using the instrument software and stored on the instrument workstation. The data file and batch file are transferred to the network where the data file is processed into a results file that is also saved on the CDC network. Results are typically generated by auto-integration, but may require in some cases manual integration. The results file (including analyte and internal standard names, peak areas, retention times, sample dilution factor, data file name, acquisition time, etc.) is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. See "**4069.03 SOP for Computerization and Data System Management**" for a step-by-step description of data transfer, review, and approval.

For NHANES, data is transmitted electronically. Abnormal values are confirmed by the analyst, and codes for missing data are entered by the analyst and are transmitted as part of the data file. NCHS makes arrangements for the abnormal report notifications to the NCHS Survey Physician.

Data files from the instrument workstation are typically copied to the CDC network on a run-by-run basis. This is the responsibility of the analyst under the guidance of the team lead and/or supervisor. Further data processing is typically conducted on a networked computer and saved directly to the CDC network. Files stored on the CDC network are automatically backed up nightly by ITSO support staff.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

We recommend that specimen donors fast prior to specimen collection, but fasting is not required.

Specimens for phytoestrogen analysis are performed on fresh or frozen urine.

3–5 mL of urine is required to allow for repeat analyses. A volume of 200 µL is required for each analysis.

The appropriate amount of urine is dispensed into a Nalgene 5.0 mL cryovial or other plastic screw-capped vial labeled with a sample ID."

Specimens collected in the field are frozen, and then shipped on dry ice by overnight carrier. Frozen samples are stored at least at -20 °C, preferably at -80 °C. Excessive freeze/thaw cycles might result in degradation of phytoestrogens in urine, however, phytoestrogens appear to be stable over the course of three freeze/thaw cycles.

Specimens generally arrive frozen. Refrigerated samples may be used provided they are kept cold and brought promptly (within 2 hours) from the site of collection.

Specimen handling conditions are outlined in the Policies and Procedures Manual of DLS (copies are available in the Nutritional Biomarkers Branch and the electronic copy of this file is located at \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\CLIA). The protocol discusses collection and transport of specimens and the special equipment required. In general, plasma should be transported and stored at no more than -20°C. Samples thawed and refrozen less than five times are not compromised. If there is more

than one test of interest in the specimen and it needs to be divided, the appropriate amount of blood or plasma should be transferred into a sterile Nalgene cryovial labeled with the participant's ID; avoid cross-contamination.

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this method.

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

A. Reagent Preparation

Prepare all reagents with 0.45 µm filtered deionized water with a resistance of at least 18 MΩ/cm, and HPLC-grade solvents and reagents. Use Class A volumetric glassware where a volumetric flask is specified. Perform all steps involving concentrated acids, bases, and organic solvents in a chemical fumehood. Although each reagent preparation specifies a total volume of reagent prepared, these directions may be scaled up or down to prepare larger or smaller quantities if desired.

1) Ammonium Acetate Buffer, pH 5.0 (2.5 M)

For 500 mL, weigh 96.25 g of ammonium acetate into a 1 L beaker and dissolve in 100 mL water. While stirring, add approximately 132 mL of glacial acetic acid. Additional glacial acetic acid or NH4OH can be added to adjust pH as needed. Transfer the solution to a 500 mL volumetric flask and fill to the mark with water. Prepare every 3 months and store at 10 °C or below.

2) β -Glucuronidase Solution

120 units of enzyme are to be added to each urine sample. Accordingly, the β –glucuronidase powder enzyme should be prepared in water at a concentration of 40 mg/mL, and allowed to dissolve (this process could take several minutes). Extreme care should be taken during this process so as not to deactivate the enzyme; do not vortex or shake vigorously. To mix, use a gentle rocking motion. Prepare daily for each run.

3) Mixed Working Deconjugation Standard Solution

Combine 800 µL of the deconjugation standard solution with 800 µL of the deconjugation internal standard solution, and thoroughly mix by vortexing. Prepare daily for each run.

4) HPLC Mobile Phase (Aqueous)

100% water. Refill as needed.

5) HPLC Mobile Phase (Organic)

100% methanol. Refill as needed.

6) HPLC Needle Wash (Organic)

75% methanol. Refill as needed.

7) Synthetic Urine

For one L, quantitatively transfer 500 mL water to a one L beaker. Using a magnetic stir bar to agitate the solution, add the following chemicals in the quantities and order specified:

a) 3.8 g Potassium Chloride

b) 8.5 g Sodium Chloride

c) 24.5 g Urea

d) 1.03 g Magnesium Sulfate (MgSO4.7H2O)

e) 1.03 g Citric Acid

f) 0.34 g Ascorbic Acid

g) 1.18 g Potassium Phosphate

h) 1.4 g Creatinine

i) 0.64 g Sodium Hydroxide (add slowly)

j) 0.47 g Sodium Bicarbonate

k) 0.28 mL Sulfuric Acid (conc.)

Once all compounds have dissolved in solution, quantitatively transfer the mixture to a one L volumetric flask. Bring the solution up to volume with water. Seal the volumetric flask and mix the contents by inversion. Transfer to a storage vessel. This solution can be stored at 4 °C for up to one year.

B. Standards Preparation

1) Analytical Standard Stock Solutions

A stock solution of each phytoestrogen standard is prepared separately by dissolving 3-5 mg of the compound in 100% ethanol and placing it in a 25 mL volumetric flask. The flask is then filled to volume with ethanol. If compounds are not dissolving, then the use of 0.2 mL of DMSO (dimethylsulfoxide) is acceptable.

2) Mixed Working Analytical Standard Solutions

Ten mixed working solutions with increasing concentration of each phytoestrogen standard are prepared in 50-mL volumetric flasks by using appropriate volumes from each standard stock solution based on the concentrations needed to cover the linear range of the assay (see **Appendix B**). Each flask is then filled to volume with the appropriate amounts of ethanol and water such that the final mixture is dissolved in 50% ethanol/water. Each mixed working solution is then dispensed in 100 µL aliquots into 1.5 mL micro-centrifuge tubes and stored upright at -80 °C.

3) Internal Standard Stock Solutions

Prepare a stock solution of each internal standard separately by adding 1-2 mg of each compound, dissolved in ethanol, to a 25 mL volumetric flask and fill to volume.

4) Mixed Working Internal Standard Solution

A mixed working internal standard solution containing the appropriate concentration of each compound (see Appendix C) is prepared by pipetting the following amounts of each internal standard stock solution into a volumetric flask of appropriate size:

$^{13}C_3$-Equol	15.79 mL
$^{13}C_3$-Daidzein	19.89 mL
$^{13}C_3$-O-Desmethylangolensin	3.13 mL
$^{13}C_3$-Genistein	15.63 mL
$^{13}C_3$-Enterolactone	15.18 mL
$^{13}C_3$-Enterodiol	12.98 mL

The flask is then filled to volume with water. The solution is dispensed in 2 mL aliquots into 2 mL Nalgene cryovials and stored at -80 °C.

5) Deconjugation Standard Solution

4-methylumbelliferyl glucuronide and 4-methylumbelliferyl sulfate are used as deconjugation standards to qualitatively determine the extent of enzymatic reaction. The deconjugation standard solution is prepared by dissolving 1.20 mg of 4-methylumbelliferyl glucuronide and 1.00 mg of 4-methylumbelliferyl sulfate in ethanol and placing in a 50 mL volumetric flask and filling to volume. The solution is then diluted with 950 mL of water (20-fold dilution) and mixed thoroughly. The diluted solution is dispensed in 1000 µL aliquots into 2 mL Nalgene cryovials and stored at -80 °C.

6) Deconjugation Internal Standard Solution

$^{13}C_4$-4-methylumbelliferone is used in conjunction with the deconjugation standard solution to qualitatively determine the extent of enzymatic reaction. 1.2-mL of a 100 µg/mL solution in acetonitrile is purchased from Cambridge Isotope Laboratories (Tewksbury, MA). The solution is diluted 100-fold and dispensed in 1000 µL aliquots into 2 mL Nalgene cryovials and stored at -80 °C.

C. Preparation of Quality Control Materials

Low, medium, and high quality control pools are prepared by selecting and pooling urine containing the appropriate levels of all six phytoestrogens. For the low pool, urine is selected that contains levels of phytoestrogens below the 25th percentile for each analyte based on currently available reference data (e.g., The Fourth National Report on Human Exposure to Environmental Chemicals **[14]**, or The Second National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population **[15]**. Urine selected for the medium pool contains levels of phytoestrogens ranging from the 25th to 75th percentile for each analyte. Urine selected for the high pool has target levels of phytoestrogens above the 75th percentile (see Appendix D for target quality control values; see Appendix E for U.S. population percentiles).

Urine (1.8 mL) is aliquoted into 2.0-mL Nalgene cryovials, capped, and frozen. The QC pools are stored at -80 °C and are stable for at least 3 years. Means plus range limits for all pools are established by analyzing duplicates for at least 20 consecutive runs.

D. Other Materials

(1) General consumables

a) Kinetex C18 analytical column, 50 x 2.1 mm, 2.6 µm (Phenomenex, Torrance, CA)

b) Krud Katcher Ultra in-line filter, 0.5 µm x 0.004" ID (Phenomenex, Torrance, CA)

c) 9" Disposable glass Pasteur pipettes (Kimble Glass, Vineland, NJ)

d) Nunc 1 mL deepwell 96-well plates (Nalge Nunc International, Rochester, NY)

e) 96-well pre-slit silicon plate seals, 8.6mm, (Thermo-Fisher Scientific, Fair Lawn, NJ)

f) 96-well filter plates, Acroprep Advanced 10K Omega, 1mL well, NTRL (Pall, Port Washingtion, NY)

g) 69-well silicon filter plate seals (Pall, Port Washingtion, NY)

h) High Five nitrile examination gloves (High Five Products Inc., Chicago, IL)

i) Blue tips (50-1000 µL) for Eppendorf pipette (Brinkmann Instruments Inc., Westbury, NY)

j) Yellow tips (2-200 µL) for Eppendorf pipettes (Brinkmann Instruments Inc., Westbury, NY)

k) Combitip plus (500 µL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)

l) Combitip plus (1.0 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)

m) Combitip plus (2.5 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)

n) Combitip plus (5.0 mL) for Eppendorf repeater pipette (Brinkmann Instruments Inc., Westbury, NY)

o) 2.0 mL Polypropylene cryovials (Nalgene Company, Rochester, NY)

p) 1.5 mL micro centrifuge tubes (VWR, Suwanee, GA)

q) 15 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)

r) 50 mL BD Falcon Tubes (Becton Dickinson, Franklin Lakes, NJ)

s) Various glass beakers, volumetric flasks, graduated cylinders, and bottles, class A glassware.

(2) Chemicals and solvents

a) Methanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)

b) Ethanol, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)

c) Dimethylsulfoxide, HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI)

d) Water, HPLC grade (Aqua Solutions, Jasper, GA)

e) Ammonium Hydroxide (28-30%, Thermo-Fisher Scientific, Fair Lawn, NJ)

f) Ammonium Acetate, HPLC grade (Sigma, St. Louis, MO)

g) Acetic acid, glacial, reagent grade (Sigma, St. Louis, MO)

h) 4-methylumbelliferyl β-D-glucuronide hydrate (Sigma, St. Louis, MO)

i) 4-methylumbelliferyl sulfate (Sigma, St. Louis, MO)

j) $^{13}C_4$-4-methylumbelliferone (Cambridge Isotope Laboratories, Tewksbury, MA)

k) β-Glucuronidase (powder), type H-1 from *Helix pomatia* (Sigma, St. Louis, MO)

l) Enterolactone (Sigma, St. Louis, MO)

m) Enterodiol (Sigma, St. Louis, MO)

n) Equol (Sigma, St. Louis, MO)

o) Genistein (Indofine Chemical Company, Somerville, NJ)

p) Daidzein (Indofine Chemical Company, Somerville, NJ)

q) O-Desmethylangolensin (Dr. Nigel Botting, University of St. Andrews, Scotland)

r) $^{13}C_3$-Enterodiol, (Dr. Nigel Botting, University of St. Andrews, Scotland)

s) $^{13}C_3$-Enterolactone, (Dr. Nigel Botting, University of St. Andrews, Scotland)

t) $^{13}C_3$-Genistein, (Dr. Nigel Botting, University of St. Andrews, Scotland)

u) $^{13}C_3$-Daidzein, (Dr. Nigel Botting, University of St. Andrews, Scotland)

v) $^{13}C_3$-Equol, (Dr. Nigel Botting, University of St. Andrews, Scotland)

w) $^{13}C_3$-O-Desmethylangolensin, (Dr. Nigel Botting, University of St. Andrews, Scotland)

E. Instrumentation

In the case of simple laboratory instrumentation (e.g., pipettes, vortex mixer, analytical balance, etc.) a product listed herein may be substituted with equivalent product from a different manufacturer provided that it meets or exceeds the specifications of the product listed. In the case of advanced laboratory instrumentation (e.g., HPLC components, tandem quadrupole mass spectrometer, automated liquid handler) equivalent performance must be demonstrated experimentally in accordance with DLS policies and procedures if a product substitution is made. Equivalent performance must also be demonstrated in accordance with DLS policies and procedures when multiple analysis systems are used in parallel, even if they are of the exact same type.

(1) Agilent 1260 (formerly 1200 SL) HPLC system (Agilent Technologies, Palo Alto, CA), including:

a) Model 4208A Control Module

b) Model G1379B Degasser

c) Model G1312B Binary pump SL

d) Model G1367D High Performance Autosampler

e) Model G1316B Thermostatted Column Compartment

(2) AB Sciex API 6500 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA), including:

a) IonDrive Turbo V ion source

b) MS table with built in nitrogen/zero air generator (Peak Scientific, Billerica, MA)

(3) Hamilton Starlet 8-channel with auto-load arm (Hamilton), including:

a) Two pipette tip carriers, TIP_CAR_480_A00

b) Three sample vial carriers, SMP_CAR-32_A00

c) One plate carrier, PLT_CAR_L5AC_A00

(4) Other laboratory instrumentation:

a) Adjustable volume pipette, F1-ClipTip, 2-20µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

b) Adjustable volume pipette, F1-ClipTip, 5-50µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

c) Adjustable volume pipette, F1-ClipTip, 10-100µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

d) Adjustable volume pipette, F1-ClipTip, 20-200µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

e) Adjustable volume pipette, F1-ClipTip, 30-300µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

f) Adjustable volume pipette, F1-ClipTip, 100-1000µL (Thermo-Fisher Scientific, Fair Lawn, NJ)

g) Vortexer (VWR)

h) Magnetic stirrer (Thermo-Fisher Scientific, Fair Lawn, NJ)

i) Economy incubator, model 3EM (Precision, Winchester, VA)

j) Analytical balance Excellence Plus XP-205 (Mettler Toledo Columbus, OH)

k) Accument XL150 pH meter (Thermo-Fisher Scientific, Fair Lawn, NJ)

7. Calibration and Calibration Verification Procedures

A. Method Calibration

Ten calibrators (S0–S9) prepared in synthetic urine are added to the reaction plate and processed as regular samples (see **Appendix B**). These 10 calibrators are analyzed at the beginning of each run. At the end of each run, the calibrators are re-analyzed as unknown samples. The measured concentrations of these calibrators should generally agree within 15% of their set values, although >15% agreement will be observed at concentrations approaching the LOD. A quadratic calibration equation with 1/x weighting is used.

Reference materials are not available for urine phytoestrogens. Calibration verification is conducted as outlined in "**4069.03 SOP for Calibration and Calibration Verification**."

External proficiency testing programs currently do not exist for urine phytoestrogens. An in-house proficiency testing program has been developed and is conducted at least twice a year, details of which can be found in "**4069.03 SOP for Alternative In-House Proficiency Testing Program**." For general information on the handling, analysis, review, and reporting of proficiency testing materials see "**NBB_SOP Proficiency Testing Procedure**."

Results from a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied are presented in **Appendix B**.

B. Instrument Calibration

1) API 6500 Mass Spectrometer

The calibration of the mass spectrometer is scheduled on an annual basis as part of a preventive maintenance program and is performed by the service engineer from AB Sciex. If necessary, the analyst can recalibrate using the calibration standards described below and by following the instructions contained in the operator's manual.

The tuning and mass calibration of the first and third quadrupoles of the API 6500 is performed using a solution of polypropylene glycol (PPG) by infusion and running the instrument in either Manual Tuning mode or using Automatic Mass Calibration. Please refer to the API 6500 User's Manual for additional details.

2) Hamilton Microlab Starlet

Twice a year a Hamilton service engineer performs a preventative maintenance including volume verification at 10 µL and 1000 µL.

A volume verification of the various steps of the method can also be performed gravimetrically (e.g., using online gravimetric kit, Hamilton) by the user. Imprecision should be commensurate or exceed that obtained using manual pipettes.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

A typical run (written here in the order in which they are injected into the LC-MS/MS) consists of 5 column equilibration injections (urine samples randomly selected from the current sample set), injection of the double blank and the blank, 10 calibrators, 3 front QCs (low, medium, and high), 78 patient samples, 3 back QCs (low, medium, and high), and lastly, reinjection of the 10 calibrators.

A. Sample Preparation

1) Manual Sample Preparation

a) Enzymatic Deconjugation

Prepare β-glucuronidase solution (described in section 6.A. of this document)

Prepare mixed working deconjugation standard solution (described in section 6.A. of this document)

Label a 96-well deepwell plate (suggested layout shown in **Appendix F**)

1. Calibrator Preparation

Add the following to each calibrator (S0-S9):

(i) 250 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)

(ii) 50 µL of mixed working deconjugation standard solution

(iii) 100 µL pH 5.0 (2.5M) ammonium acetate buffer

(iv) 900 µL synthetic urine

(v) 50 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

Thoroughly mix each calibrator. Transfer 290 µL of each calibrator (S0-S9) to the appropriate well, as labeled on the 96-well plate. Discard remaining calibrator solution.

2. Double Blank Preparation

(i) Add the following to the well labeled "double blank" on the 96-well plate:

(ii) 10 µL of mixed working deconjugation standard solution

(iii) 20 µL pH 5.0 (2.5M) ammonium acetate buffer

(iv) 250 µL synthetic urine (no urine or mixed working internal standard solution present in the double blank)

(v) 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

3. Blank Preparation

Add the following to the well labeled "blank" on the 96-well plate:

(i) 50 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)

(ii) 10 µL of mixed working deconjugation standard solution

(iii) 20 µL pH 5.0 (2.5M) ammonium acetate buffer

(iv) 200 µL synthetic urine (no urine present in the blank)

(v) 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

4. Urine Sample Preparation (quality control materials and unknown samples)

Add the following to all wells labeled "QC" and "Unknowns" on the 96-well plate:

(i) 50 µL of mixed working internal standard solution, diluted 5x (1 part IS solution, 4 parts water)

(ii) 10 µL of mixed working deconjugation standard solution

(iii) 20 µL pH 5.0 (2.5M) ammonium acetate buffer

(iv) 200 µL urine

(v) 10 µL of β-glucuronidase solution (IMPORTANT – add enzyme last)

Place pre-slit silicone plate mat on 96-well plate and mix gently by hand (not vigorously and do not vortex as this may cause deactivation of the enzyme), making sure that all contents are washed from the walls of each well. Incubate overnight (at least 12 hours) at 45 ° ± 2 °C.

b) Filtration

The filtration procedure applies to all sample types [calibrators, double blank, blank, QC's, unknowns], after incubation).

Add 150 µL of HPLC-grade methanol to each sample and mix thoroughly.

Transfer 300 µL of each sample (all specimen types: calibrators, blanks, QC's, and unknowns) to a 96-well filter plate, such that the positions correspond to that of the sample preparation 96-well plate.

Place a new, clean 96-well plate (same type as used during sample preparation) under the filter plate to serve as a collection plate, and centrifuge at 3000xg for 1 hour. IMPORTANT – be sure to place an additional filter plate (containing water only) and corresponding collection plate in the centrifuge at the same time to serve as a counterweight.

After centrifugation, place a new, clean silicone plate mat on the 96-well plate used for collection and place in the HPLC autosampler for LC-MS/MS analysis.

2) Automated Sample Preparation

"**4069.03 SOP for Automated Urine Aliquoting**" and "**4069.03 SOP for Automated Methanol Aliquoting (Pall or Millipore**)" describe automated sample preparation steps using the Hamilton Starlet system. These steps directly mimic those described above for manual sample preparation with most pipetting actions being performed by the Hamilton Starlet.

The instructions given in the SOP reflect the custom program developed for performing sample preparation that is currently being used. Certain non-critical elements of this program (e.g., positions of samples, wording of user messages) may be modified and differ from the exact instructions given in the SOP. The user is strongly encouraged to be familiar with the exact program being used.

A liquid handling system other than the Hamilton Starlet may be used for this purpose provided that it is able to perform these steps with accuracy and precision that meets or exceeds that of the Hamilton Starlet.

B. Instrument Preparation

1) HPLC Preparation

Solvent bottles should be checked daily and refilled as needed. Line A1 contains aqueous HPLC mobile phase, line B1 contains organic HPLC mobile phase, and a line coming from the auto-sampler contains organic needle wash (described in section 6.A. of this document). The waste bottle should be checked daily to ensure that it will not overflow during the run.

Phenomenex Krud Katcher Ultra in-line filter, 0.5µm x 0.004" ID, should be replaced as needed.

Before each run, review the chromatographic spectra of the previous runs' calibrators to ensure that the Phenomenex Kinetex C18 analytical column (50x2.1mm, 2.6µm) is in suitable condition (i.e. no double peaking, peak trailing, broad peaks, etc.). Replace the analytical column as needed.

2) Mass Spectrometer Preparation

Check the interface settings before each run to make sure the probe height and width settings are correct.

Clean the interface as needed by removing the interface housing (caution, if the instrument is in ready mode the housing will be very hot), and curtain plate followed by wiping the curtain plate and orifice plate with water and then methanol. Also clean out the source using water and methanol as needed.

Using the gauges on the gas generator, verify that the source gas, curtain gas, and exhaust gas pressures meet or exceed the recommended specifications (see below) set by the manufacturer.

- Source gas: 100 psi
- Curtain gas: 60 psi
- Exhaust gas: 50 psi

C. Sample Analysis

The HPLC-MS/MS system is used to quantitate phytoestrogen levels in urine. See "**4069.03 SOP for Sample Analysis**" for a detailed description of the sample analysis steps. Additional LC-ESI-MS/MS parameters are contained in **Appendix G** (gradient information) and **Appendix H** (example MS parameters). The following is an overview of the sample analysis process.

1) Preliminaries

The user must first ensure that all instrumentation is turned on and ready for use. This entails starting Analyst software and ensuring the correct project and hardware configuration is selected and activated. Refer to "**4069.03 SOP for Sample Analysis**" for additional details.

2) Building an Acquisition Batch

Because of the number of steps involved in building a new batch file, it is acceptable for the user to use a previous batch file and modify it to suit the current analysis by changing the necessary information (e.g., sample names, sample IDs, data file names, comments, etc..). In brief, the analyst must create sample sets to accommodate the following: the startup methods; equilibration injections; analysis of calibrators, QC and unknown samples; and shutdown methods. These sample sets should be run in the order presented above. Refer to "**4069.03 SOP for Sample Analysis**" for additional details.

3) Instrument Equilibration

The instrument should be equilibrated for approximately 30 minutes prior to starting an analysis. Though instrument equilibration is presented following the building of the acquisition batch, the acquisition batch can be built while the instrument is equilibrating.

This procedure assumes that the user is starting a new analysis after the instrument has successfully completed a previous analysis. The user may deviate from this procedure if special circumstances present themselves (e.g., restarting an instrument run that was interrupted). Refer to "**4069.03 SOP for Sample Analysis**" for additional details.

4) Submitting and Starting a Batch

Once the instrument has been properly equilibrated and the acquisition batch has been created and saved, the user may submit the batch to the analysis queue and start the analysis sequence. Refer to "**4069.03 SOP for Sample Analysis**" for additional details.

D. Quantification and Data Review

The quantitation of instrument results can be done either at the instrument computer or a different location (e.g., desktop PC) where the LC-MS/MS software is installed. In order to review data at a location other than the instrument, the user will have to create an identical project and copy all required files over to this location.

The following instructions assume that a complete analyses of samples in negative ion mode was performed. Refer to "**4069.03 SOP for Quantitation and Data Review**" for additional details.

(1) Create a Results Table

A data file will be created for each sample set submitted for analysis. The user will have to create a results file for each data file being processed. This will typically be the done only for the analysis sets of samples in negative ion mode. Refer to "**4069.03 SOP for Quantitation and Data Review**" for additional details.

(2) Review Peak Integration

The quantitation method is set up to identify and integrate analyte and internal standard peaks based on specifications such as retention time windows and minimum peak area thresholds. The user should review all peak integrations and correct any integration errors where necessary. Refer to "**4069.03 SOP for Quantitation and Data Review**" for additional details.

(3) Review Calibration Curves

The analyst should review the calibration curve for each analyte, ensuring that the correct regression model and weighting are used in each case. If a calibration point appears to be erroneous, it may be removed from the curve in consultation with the team lead (Note: the analyst should be aware of the implications of removing the highest or lowest calibration point as this may affect the reportable range of values for an instrument run). Refer to "**4069.03 SOP for Quantitation and Data Review**" for additional details.

(4) Importing Results into LIMS Database

The results file is imported into a LIMS database for review of the patient data, statistical evaluation of the QC data, and approval of the results. Refer to "**4069.03 SOP for Computerization and Data System Management**" for additional details.

E. System Maintenance

(1) Hamilton Microlab Starlet – Preventative Maintenance is performed semi-annually by the service engineer.

(2) Agilent 1260 HPLC – Preventative Maintenance is performed annually by the service engineer.

(3) AB Sciex API 6500 Mass Spectrometer – Preventative maintenance and tuning and calibration of the instrument is performed annually by the service engineer.

F. CDC Modifications

N/A. This manuscript is an adaptation of an original method **[14]** and a technical note is in preparation to be published in a peer-reviewed journal.

9. Reportable Range of Results (AMR – Analytical Measurement Range)

The reportable range of results, defined as the concentration range between the LOD and the highest calibrator (S9), for each of the six phytoestrogens is as follows:

Analyte	Reportable Range (ng/mL)
Equol	0.1–100
Daidzein	0.4–1,600
O-Desmethylangolensin	0.1–300
Genistein	0.2–730
Enterolactone	0.2–3,300
Enterodiol	0.09–320

Samples with concentrations less than the lowest calibrator are not reported. Also, samples with concentrations exceeding the highest calibrator are diluted, re-prepared, and reanalyzed so that the measured value is within the range of the calibration. There is no known maximum acceptable dilution. When possible, avoid small volume pipetting and minimize use of serial dilutions when generating diluted samples.

10. Quality Control (QC) Procedures

A. Blind Quality Controls

Blind QC specimens are inserted prior to the arrival of the samples in the Nutritional Biomarkers Branch. These specimens are prepared at two levels so as to emulate the patient samples; the labels used are identical to those used for patient samples. One blind QC specimen randomly selected for concentration is included at a randomly selected location in every 20 specimens analyzed.

Alternatively, open label blind QC specimens can be used where the analyst knows that the sample is a blind QC, but they do not know what pool the sample is from. Open label blind QCs are used only if they can be selected from at least 6 different pools and the analyte concentrations are similar to those found in patient samples.

B. Bench Quality Controls

Bench QC specimens are prepared from three urine pools that represent low, medium and high levels of urine phytoestrogens. Samples from these pools are prepared in the same manner as patient samples and analyzed in duplicate as part of each run.

The results from the pools are checked after each run using a multi-rule quality control system **[16]** based their characterization data, namely: the pool mean; the pooled within-run standard deviation associated with individual QC results measured in the same run (S_w); the standard deviation associated with individual QC results (S_i); and the standard deviation associated with run mean QC results (S_m). QC rules have been designed to accommodate the use of 1–3 different QC pools during a run, the use of 1–2 measurements of each pool per run, and as many instruments as needed. In the case of three QC pools per run with two QC results per pool:

(1) If all three QC run means are within 2 S_m limits and individual results are within 2 S_i limits, accept the run.

(2) If one of the three QC run means is outside a 2 S_m limit, reject run if:

a) 1 3S Rule—run mean is outside a 3 S_m limit or

b) 2 2S Rule—two or more of the three run means are outside the same 2 S_m limit or

c) 10 X-bar Rule—current and previous nine run means are on the same side of the characterization mean

(3) If one of the six QC individual results is outside a 2 S_i limit, reject run if:

a) Outlier—one individual result is beyond the characterization mean ± 4 S_i or

b) R 4S Rule—two or more of the within-run ranges in the same run exceed 4 S_w (i.e. 95 per cent range limit).

A QC program written in SAS is available from the DLS Quality Assurance Officer and should be used to apply these rules to QC data and generate Shewhart QC charts. No results for a given analyte are to be reported from an analytical run that has been declared "out of control" for that analyte as assessed by internal (bench) QC.

The initial limits are established by analyzing pool material in 20 consecutive runs and then are reevaluated periodically. When necessary, limits are updated to include more runs.

While a study is in progress, QC results are stored in the STARLIMS database. For runs that are not imported into STARLIMS (exception, research-type runs), QC results are stored electronically in the analyte-specific folder on \\cdc\project\CCEHIP_NCEH_DLS_NBB_LABS\Data handling\QC.

C. Sample QC Criteria

Each individual Sample result is checked against established sample QC criteria limits to assure data quality. The method uses the following sample QC criteria:

- Umbelliferone (UMB) area ratio
- Confirmation ion ratio (confirmation ion area/quantitation ion area)

For additional details and criteria, see "**4069.03 SOP Sample QC Criteria**"

11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

A general guideline for identifying and resolving possible problems resulting in "out of control" values for QC materials can be found in "**4069.03 SOP for OOC Corrective Action**." The troubleshooting process should be done in consultation with the supervisor and may involve additional experiments beyond what is indicated below. Analytical results for runs not in statistical control should not be reported.

(A) Check sensitivity of instrument

(B) Look for sample preparation errors, e.g., if the analyst forgot to add internal standard, specimen, etc.

(C) Check the proper gas flow for curtain, exhaust, and source from the nitrogen generator.

(D) Run standards in Q1 Scan to see if molecular ion is detected.

(E) Check to make sure that the hardware is functioning properly. Make sure the Mass spectrometer calibrations are proper. Run PPGs in Q1 Scan to check the instrument calibration.

(F) Check the calibrations of the pipettes.

12. Limitations of Method; Interfering Substances and Conditions

The most common cause of poor method performance is a pipetting error. All reagents and mobile phases should be made fresh whenever possible and verified for performance. Occasionally, the concentration of phytoestrogens in urine will exceed the highest calibrator. In this case, a smaller aliquot of urine can be used as described earlier. When using a quadratic equation for calibration, care must be taken to minimize excessive "roll-over" of the curve at higher concentrations. This phenomenon is typically indicative of too much analyte being injected. If it is observed, reducing the sample injection volume is recommended.

This method has also undergone a series of in-house ruggedness testing experiments designed to assess how much method accuracy changes when certain experimental parameters are varied. A total of five parameters judged to most likely affect the accuracy of the method have been identified and tested. Testing generally consisted of performing replicate measurements on a test specimen with the selected parameter set at a value substantially lower and higher than that specified in this method while holding all other experimental variables constant. The ruggedness testing findings for this method are presented in **Appendix E**. Please refer to Chapter 20 of the 2017 *DLS Policies and Procedures Manual* for further information on ruggedness testing.

13. Reference Ranges (Normal Values)

Refer to **Appendix E**.

14. Critical Call Results ("Panic Values")

There are no established critical values for urine phytoestrogens, i.e. there is no definition of a safe, normal or acceptable concentration of urine phytoestrogens versus one that would be considered abnormal or life-threatening.

15. Specimen Storage and Handling during Testing

Urine samples may be stored overnight in the refrigerator to expedite thawing prior to aliquotting. Samples should be allowed to warm to and be maintained at room temperature during preparation and testing and then returned to frozen storage (typically at ≤-70 °C) as soon as possible.

16. Alternate Methods for Performing Test of Storing Specimens if Test System Fails

There are no acceptable alternative methods for the analysis of phytoestrogens in the Nutritional Biomarkers Branch. If the analytical system fails, we recommend that the specimens or prepared samples be stored (typically at ≤-70 °C) until the analytical system is restored to functionality.

17. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Test results are reported to the collaborating agency at a frequency and by a method determined by the study coordinator. Generally, data from this analysis are compiled with results from other analyses and sent to the responsible person at the collaborating agency as an Excel file, either through electronic mail or on a diskette.

For NHANES 1999+, all data are reported electronically on a periodic basis to Westat and then are transferred to NCHS. For smaller studies, electronic copies of a data report are sent; a hard copy of the data report may also be sent if requested.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

The LIMS is used to keep records and track specimens for all studies. For studies other than NHANES, additional records may be kept in Excel files on the network.

We recommend that records, including related QA/QC data, be maintained for 10 years after completion of the NHANES study. Only numerical identifiers should be used (e.g., case ID numbers). All personal identifiers should be available only to the medical supervisor or project coordinator. Residual urine from these analyses for non-NHANES studies are retained for at least 1 year after results have been reported and may then be returned or discarded at the request of the principal investigator. Very little residual material will be available after NHANES analyses are completed, however residual urine is retained for at least 2 years after results have been publicly released; at that point, samples with sufficient volume (>0.2 mL) are returned to NHANES and samples with insufficient may be autoclaved.

The exact procedure used to track specimens varies with each study and is specified in the study protocol or the interagency agreement for the study. Copies of these documents are kept by the supervisor. In general, when specimens are received, the specimen ID number is entered into a database and the specimens stored in a freezer at -80 °C. The specimen ID is read off of the vial by a barcode reader used to prepare the electronic specimen table for the analytical system. When the analyses are completed, the results file is loaded into the database, and the analytical results are linked to the database by ID number. The analyst is responsible for documenting and keeping a record of specimens prepared incorrectly, those with labeling problems, and those with abnormal results, together with information about these discrepancies. In general, these are documented using codes in LIMS.

19. Method Performance Documentation

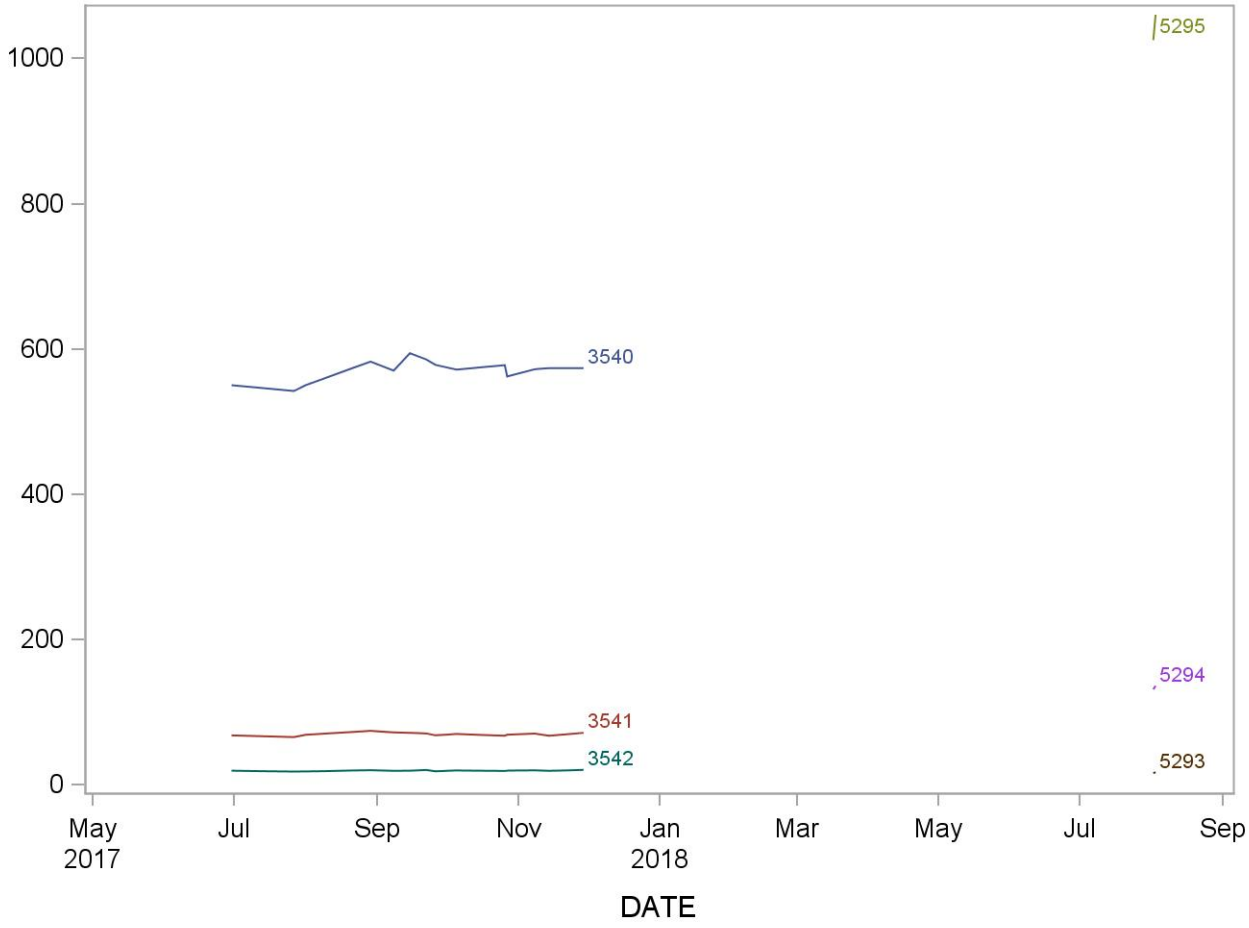
Method performance documentation for this method including accuracy, precision, sensitivity, specificity and stability is provided in **Appendix A** of this method documentation. **The signatures of the branch chief and director of the Division of Laboratory Sciences on the first page of this procedure denote that the method performance is fit for the intended use of the method.**

20. Summary Statistics and QC Graph

Please see following pages.

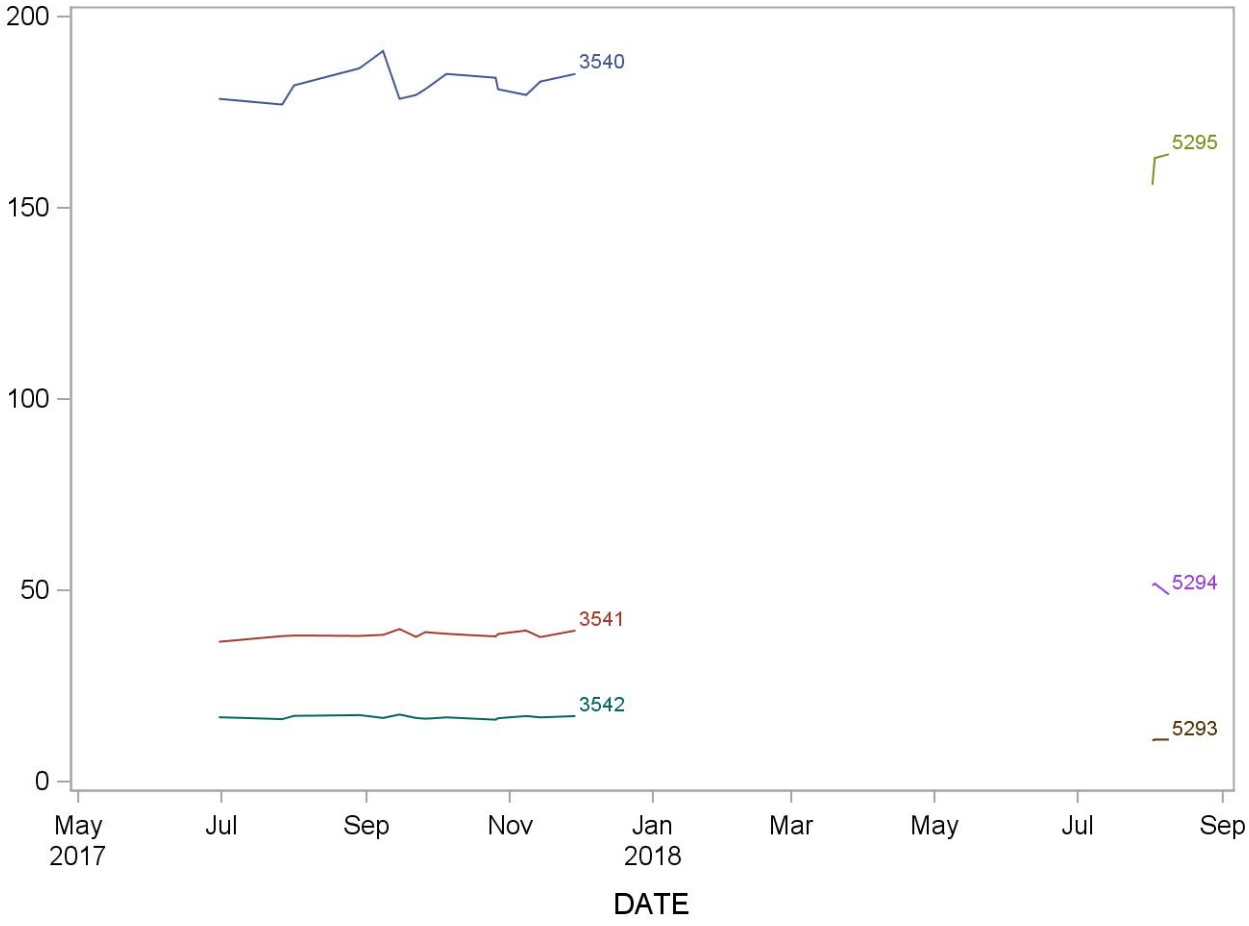
2013-2014 Summary Statistics and QC Chart for Daidzein, Urine 1st collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	570.14	14.60	2.6
3541	14	30JUN17	29NOV17	69.49	2.26	3.3
3542	14	30JUN17	29NOV17	19.21	0.71	3.7
5293	2	02AUG18	03AUG18	16.50	0.92	5.6
5294	2	02AUG18	03AUG18	133.60	3.39	2.5
5295	2	02AUG18	03AUG18	1042.50	24.75	2.4



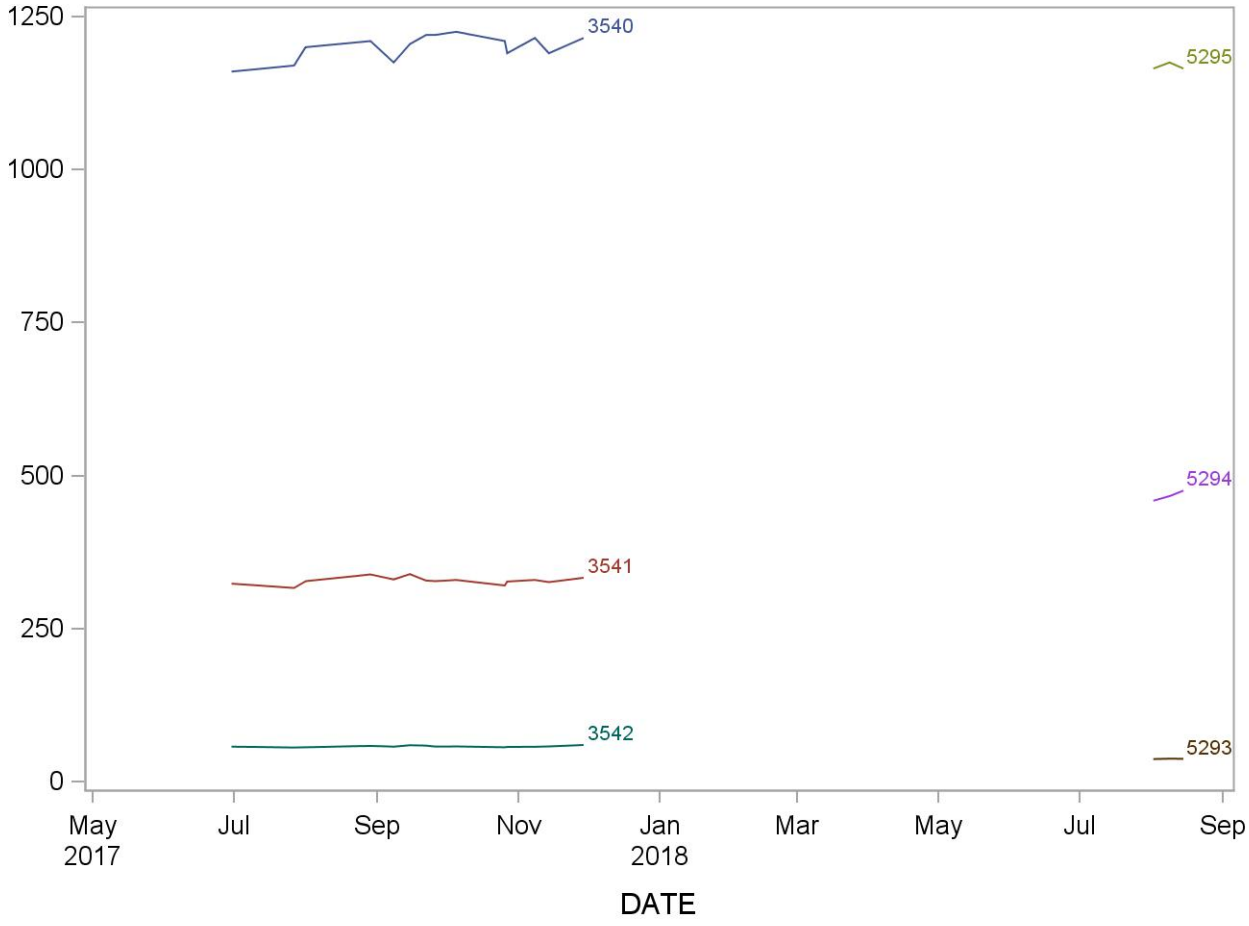
2013-2014 Summary Statistics and QC Chart for Enterodiol, Urine 1st Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	182.25	3.80	2.1
3541	14	30JUN17	29NOV17	38.44	0.87	2.3
3542	14	30JUN17	29NOV17	16.84	0.40	2.4
5293	3	02AUG18	09AUG18	10.93	0.12	1.1
5294	3	02AUG18	09AUG18	50.67	1.51	3.0
5295	3	02AUG18	09AUG18	161.00	4.36	2.7



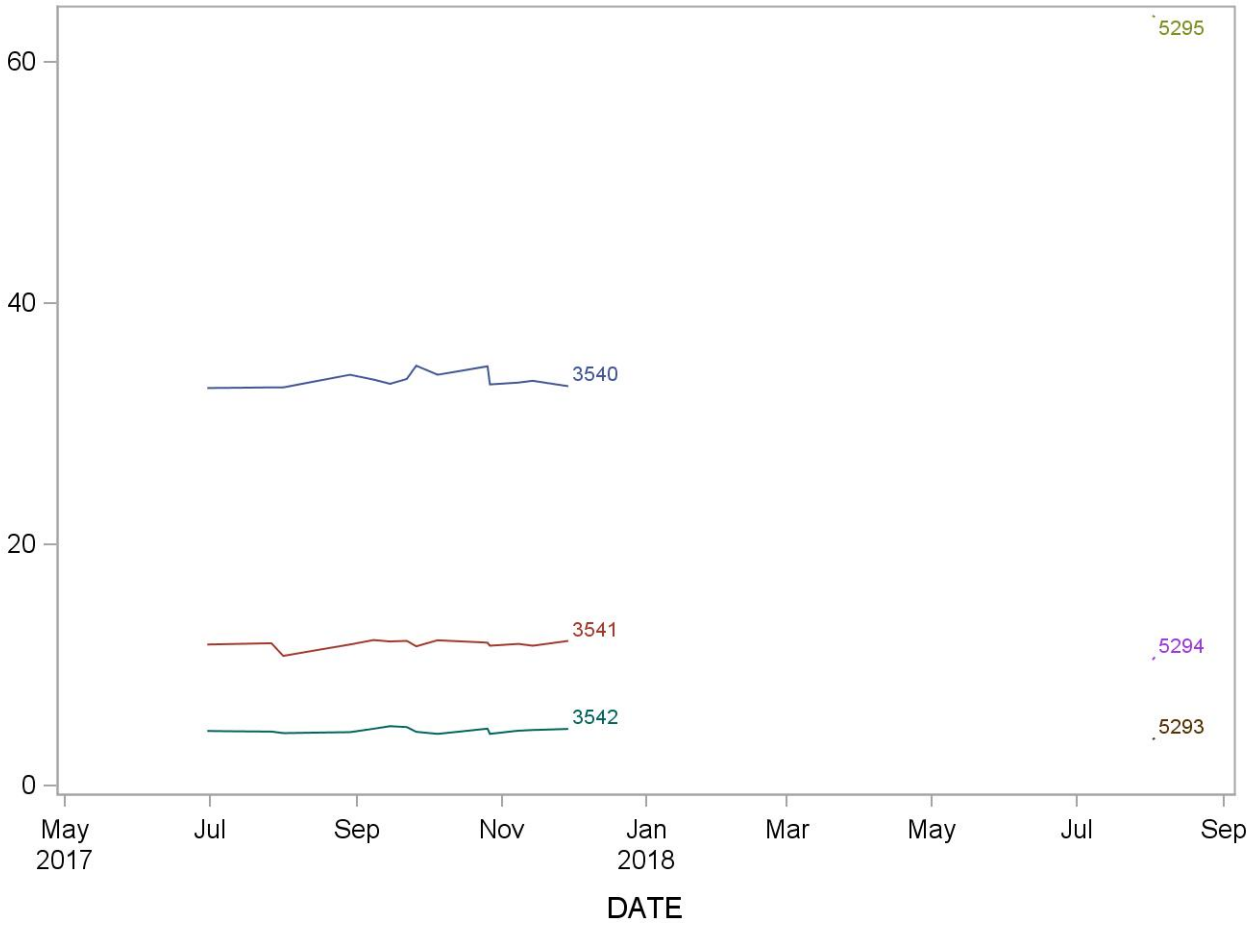
2013-2014 Summary Statistics and QC Chart for Enterolactone, Urine 1st Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	1200.357	20.425	1.7
3541	14	30JUN17	29NOV17	328.345	6.070	1.8
3542	14	30JUN17	29NOV17	57.529	1.262	2.2
5293	3	02AUG18	15AUG18	37.300	0.265	0.7
5294	3	02AUG18	15AUG18	467.000	8.261	1.8
5295	3	02AUG18	15AUG18	1168.333	5.774	0.5



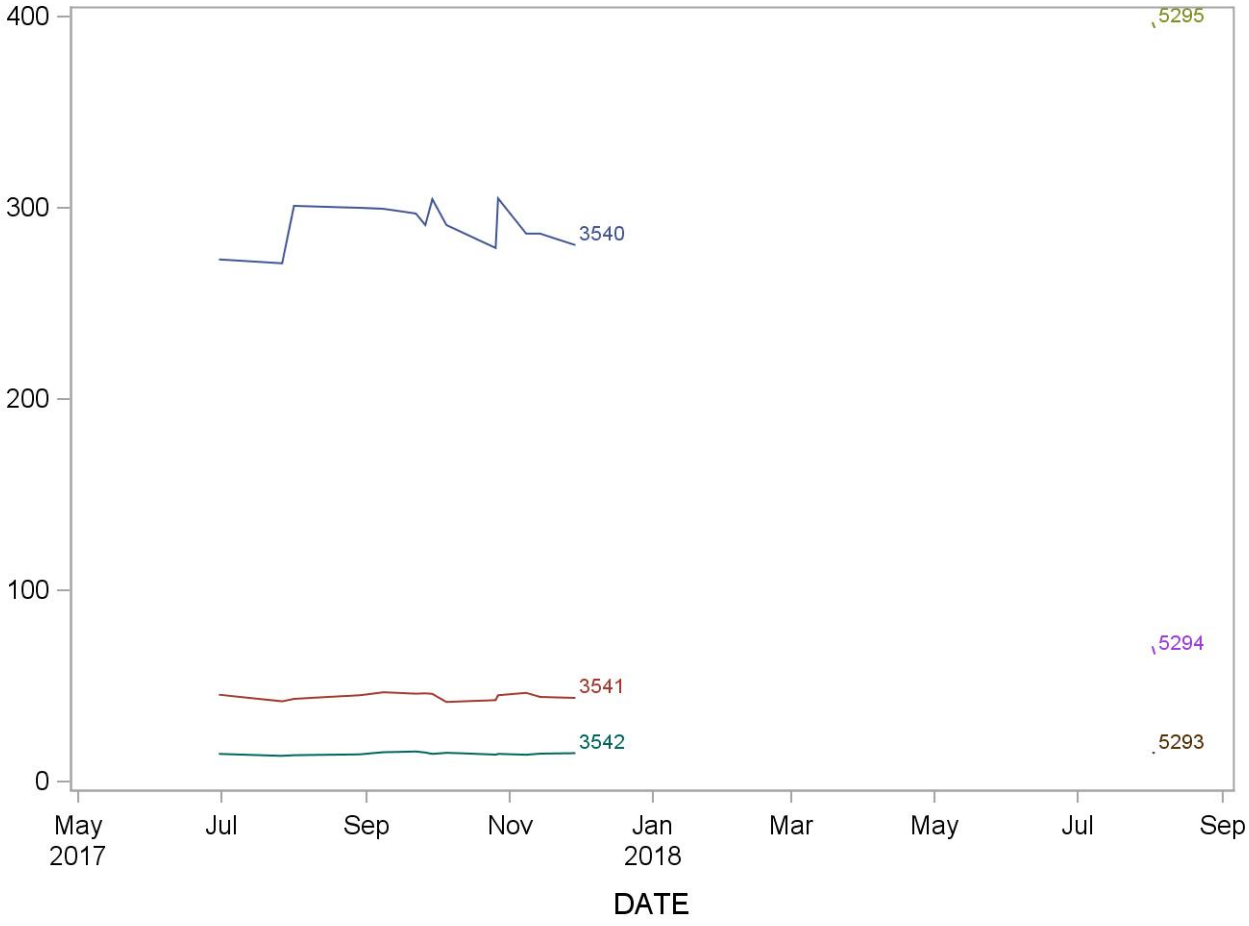
2013-2014 Summary Statistics and QC Chart for Equol, Urine 1st Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	33.61	0.61	1.8
3541	14	30JUN17	29NOV17	11.74	0.33	2.9
3542	14	30JUN17	29NOV17	4.57	0.20	4.4
5293	2	02AUG18	03AUG18	3.89	0.06	1.6
5294	2	02AUG18	03AUG18	10.54	0.15	1.5
5295	2	02AUG18	03AUG18	63.75	0.07	0.1



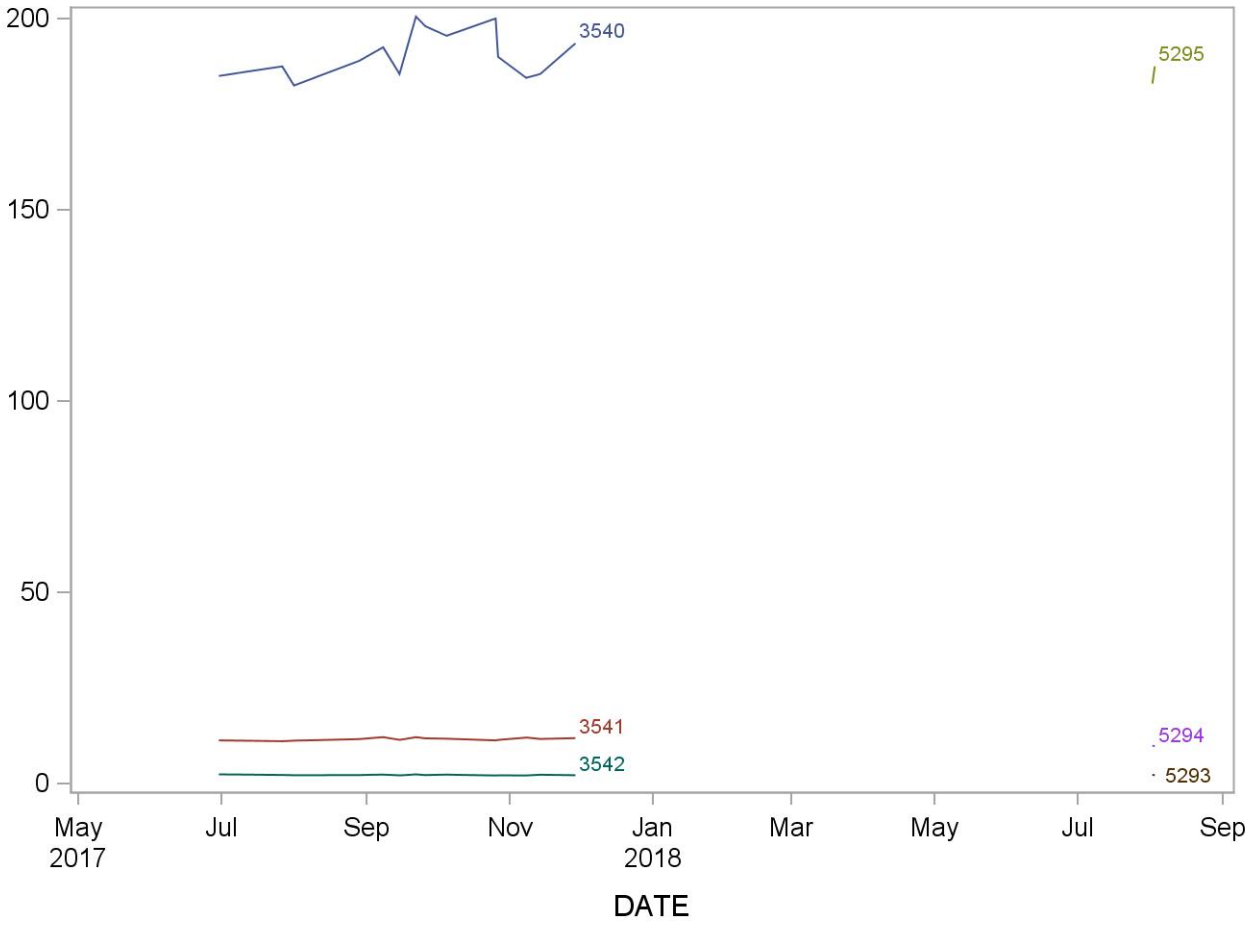
2013-2014 Summary Statistics and QC Chart for Genistein, Urine 1st Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	290.393	11.372	3.9
3541	14	30JUN17	29NOV17	44.613	1.688	3.8
3542	14	30JUN17	29NOV17	14.600	0.622	4.3
5293	2	02AUG18	03AUG18	15.075	0.035	0.2
5294	2	02AUG18	03AUG18	68.673	2.979	4.3
5295	2	02AUG18	03AUG18	395.500	2.121	0.5



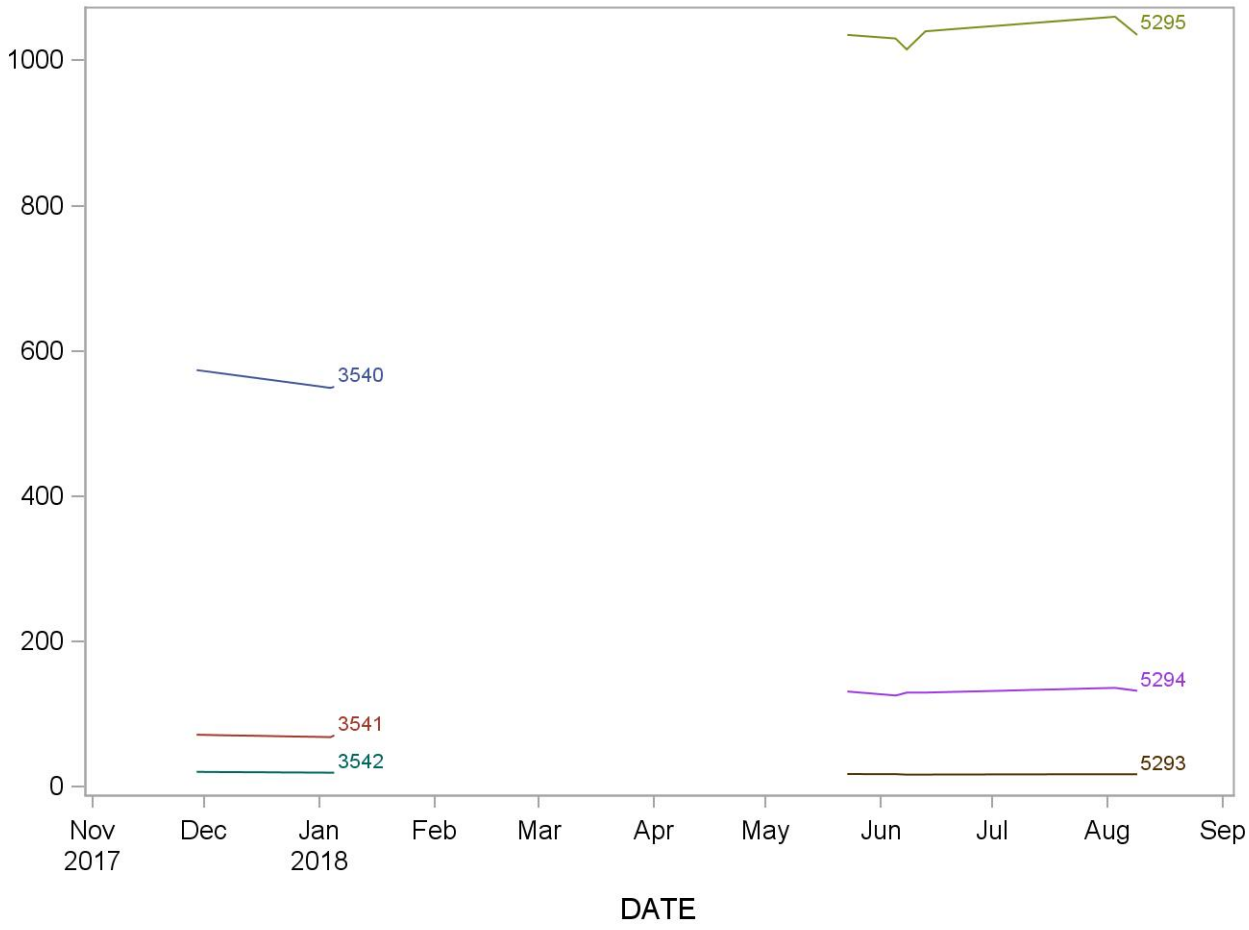
2013-2014 Summary Statistics and QC Chart for o-Desmethylangolensin, Urine 1st Collect (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	14	30JUN17	29NOV17	190.679	6.050	3.2
3541	14	30JUN17	29NOV17	11.648	0.349	3.0
3542	14	30JUN17	29NOV17	2.265	0.101	4.5
5293	2	02AUG18	03AUG18	2.260	0.106	4.7
5294	2	02AUG18	03AUG18	9.862	0.108	1.1
5295	2	02AUG18	03AUG18	185.250	3.182	1.7



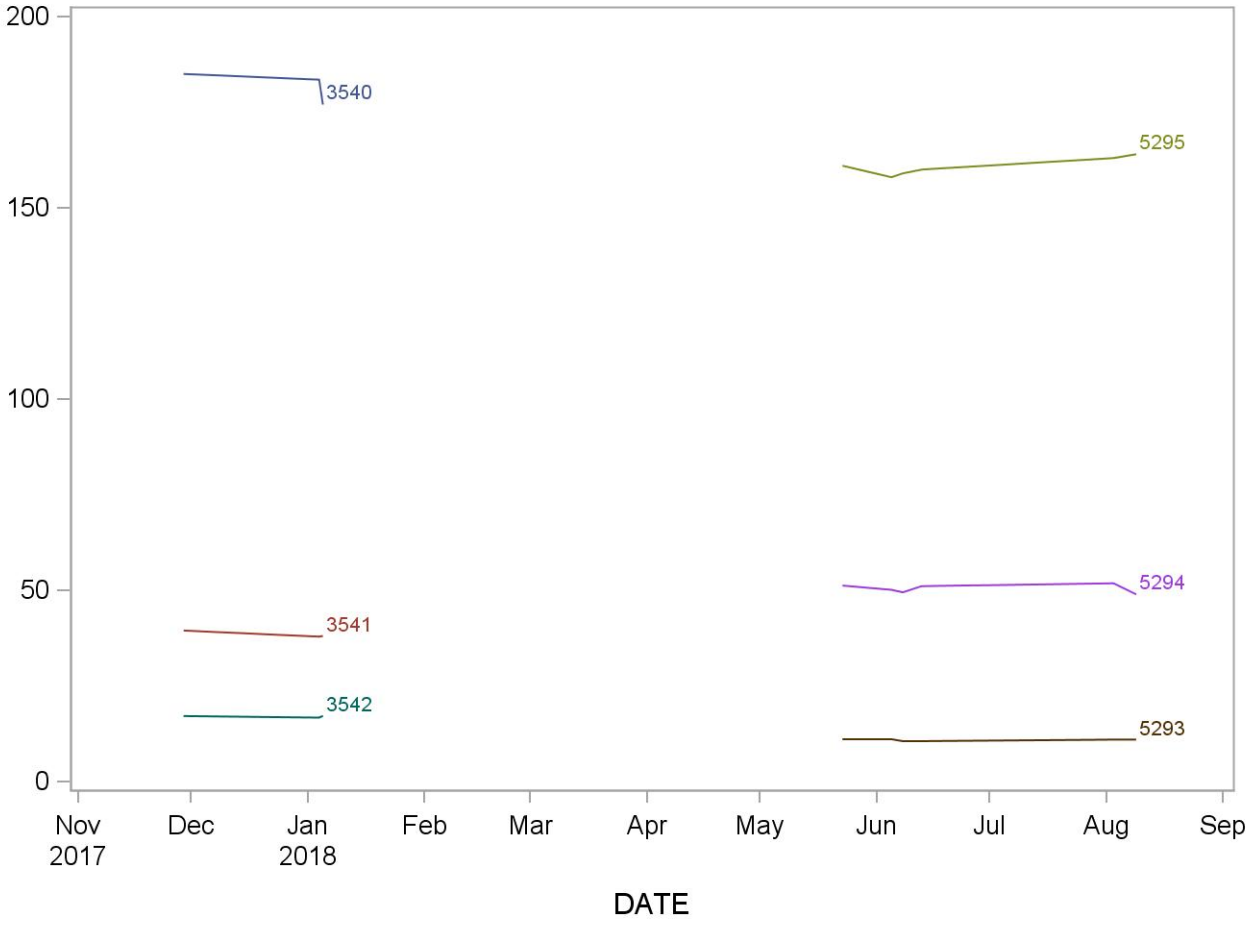
2013-2014 Summary Statistics and QC Chart for Daidzein, Urine 2nd collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	557.67	13.73	2.5
3541	3	29NOV17	05JAN18	70.07	1.67	2.4
3542	3	29NOV17	05JAN18	19.62	0.59	3.0
5293	6	23MAY18	09AUG18	17.01	0.33	2.0
5294	6	23MAY18	09AUG18	130.58	3.46	2.6
5295	6	23MAY18	09AUG18	1035.83	14.63	1.4



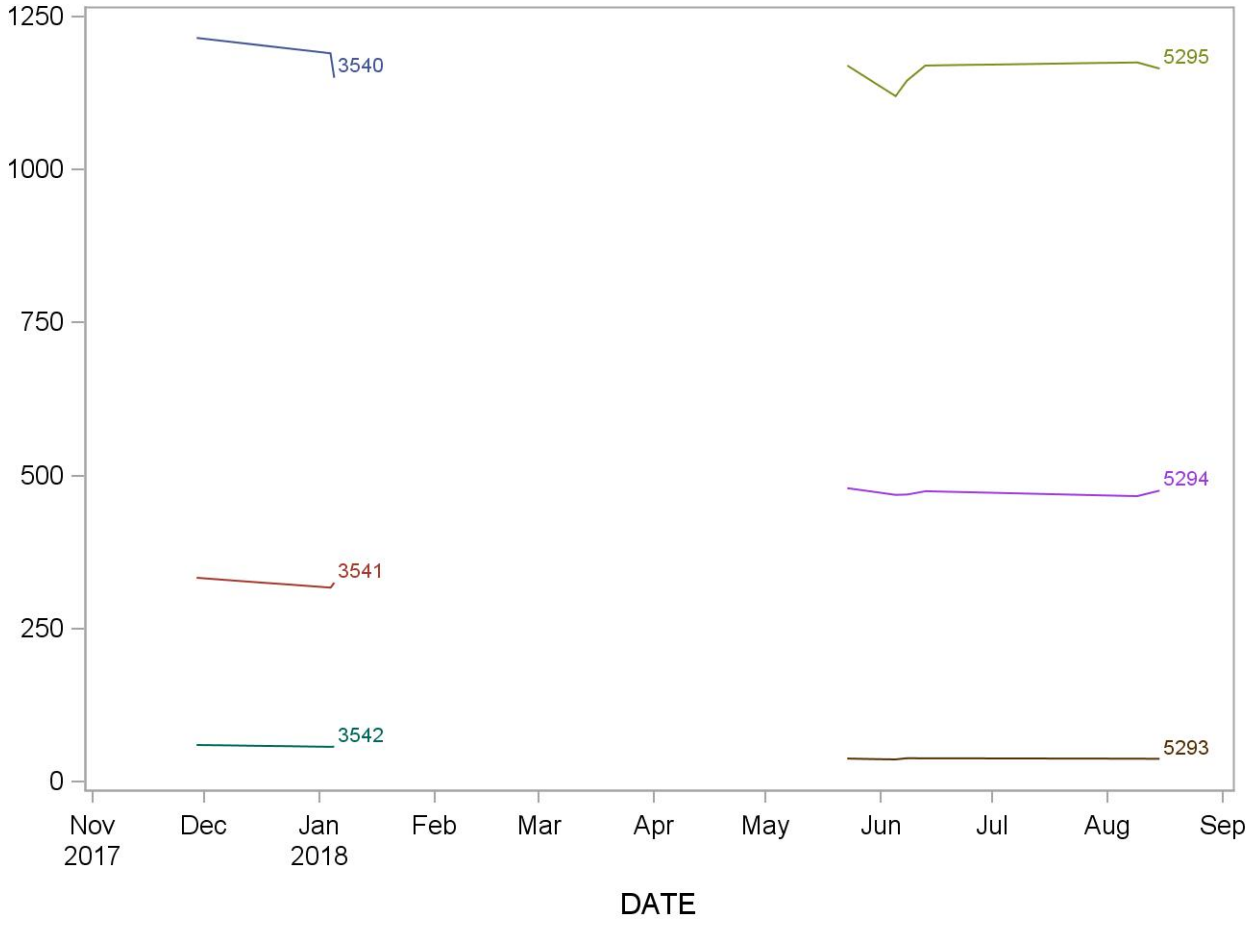
2013-2014 Summary Statistics and QC Chart for Enterodiol, Urine 2nd Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	181.83	4.25	2.3
3541	3	29NOV17	05JAN18	38.50	0.87	2.3
3542	3	29NOV17	05JAN18	17.03	0.25	1.4
5293	6	23MAY18	09AUG18	10.90	0.24	2.2
5294	6	23MAY18	09AUG18	50.47	1.12	2.2
5295	6	23MAY18	09AUG18	160.83	2.32	1.4



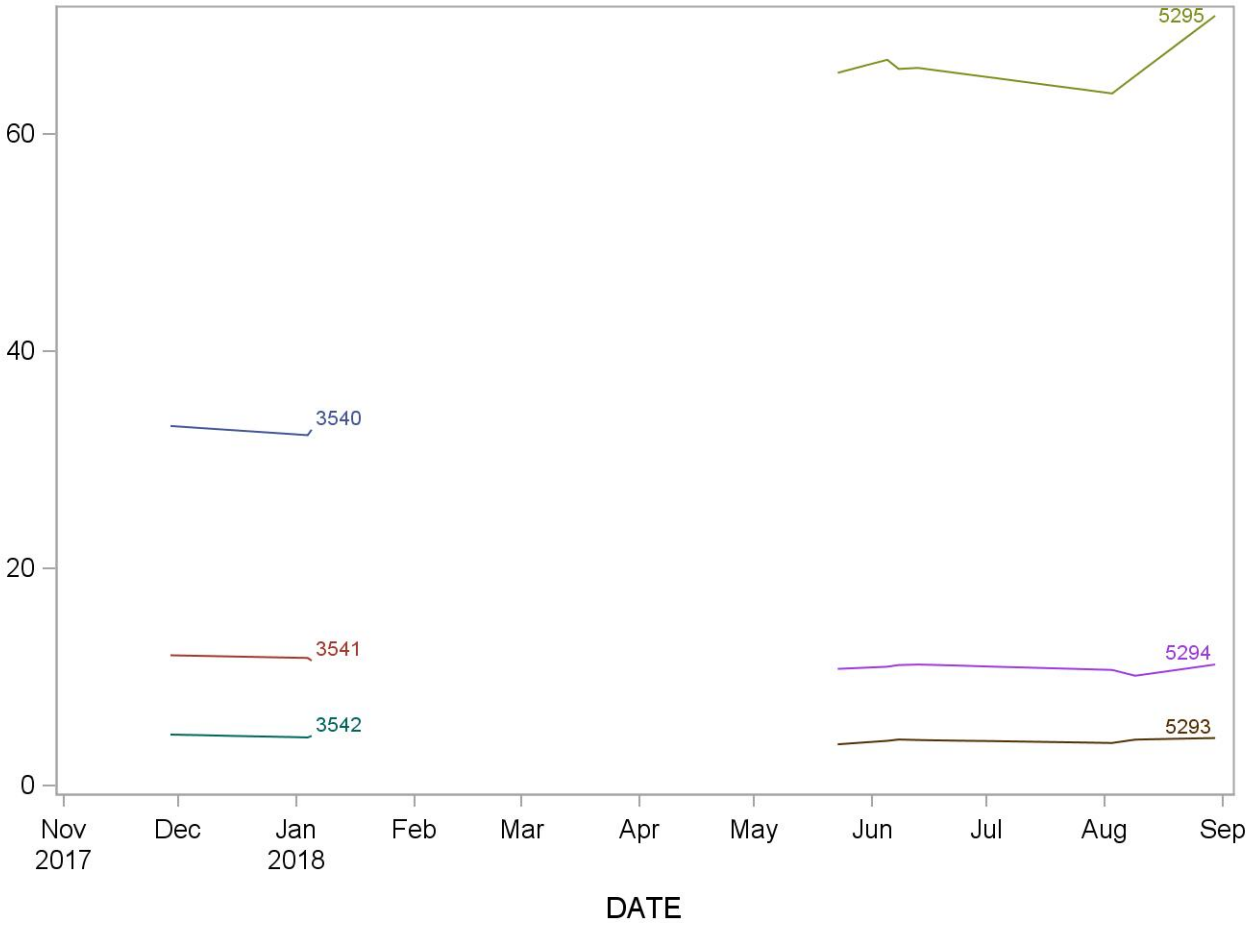
2013-2014 Summary Statistics and QC Chart for Enterolactone, Urine 2nd Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	1185.000	32.787	2.8
3541	3	29NOV17	05JAN18	325.000	8.000	2.5
3542	3	29NOV17	05JAN18	57.983	1.706	2.9
5293	6	23MAY18	15AUG18	37.550	0.709	1.9
5294	6	23MAY18	15AUG18	472.250	5.017	1.1
5295	6	23MAY18	15AUG18	1157.500	21.154	1.8



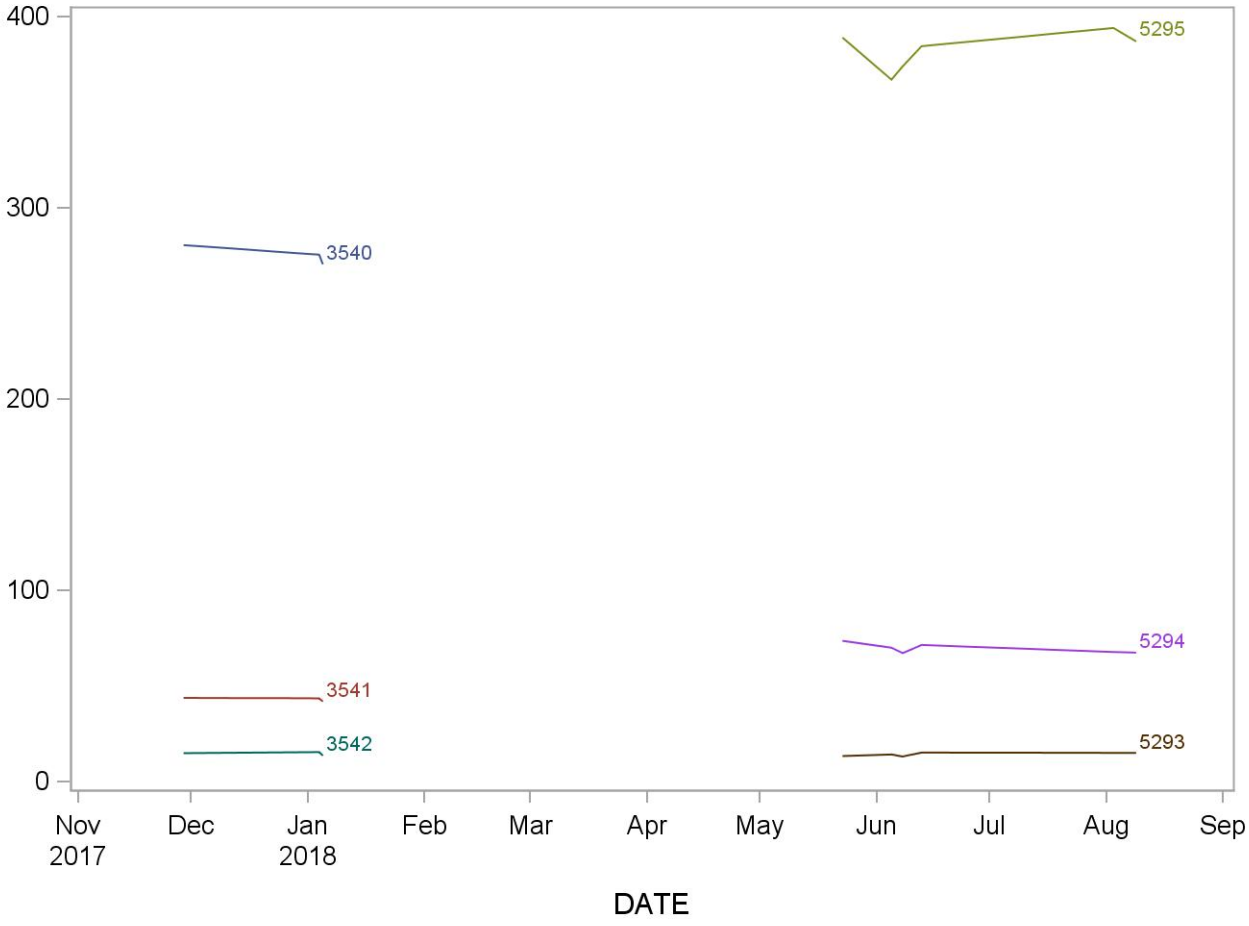
2013-2014 Summary Statistics and QC Chart for Equol, Urine 2nd Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	32.70	0.43	1.3
3541	3	29NOV17	05JAN18	11.75	0.25	2.1
3542	3	29NOV17	05JAN18	4.57	0.14	3.0
5293	7	23MAY18	30AUG18	4.13	0.20	4.9
5294	7	23MAY18	30AUG18	10.84	0.37	3.5
5295	7	23MAY18	30AUG18	66.32	2.21	3.3



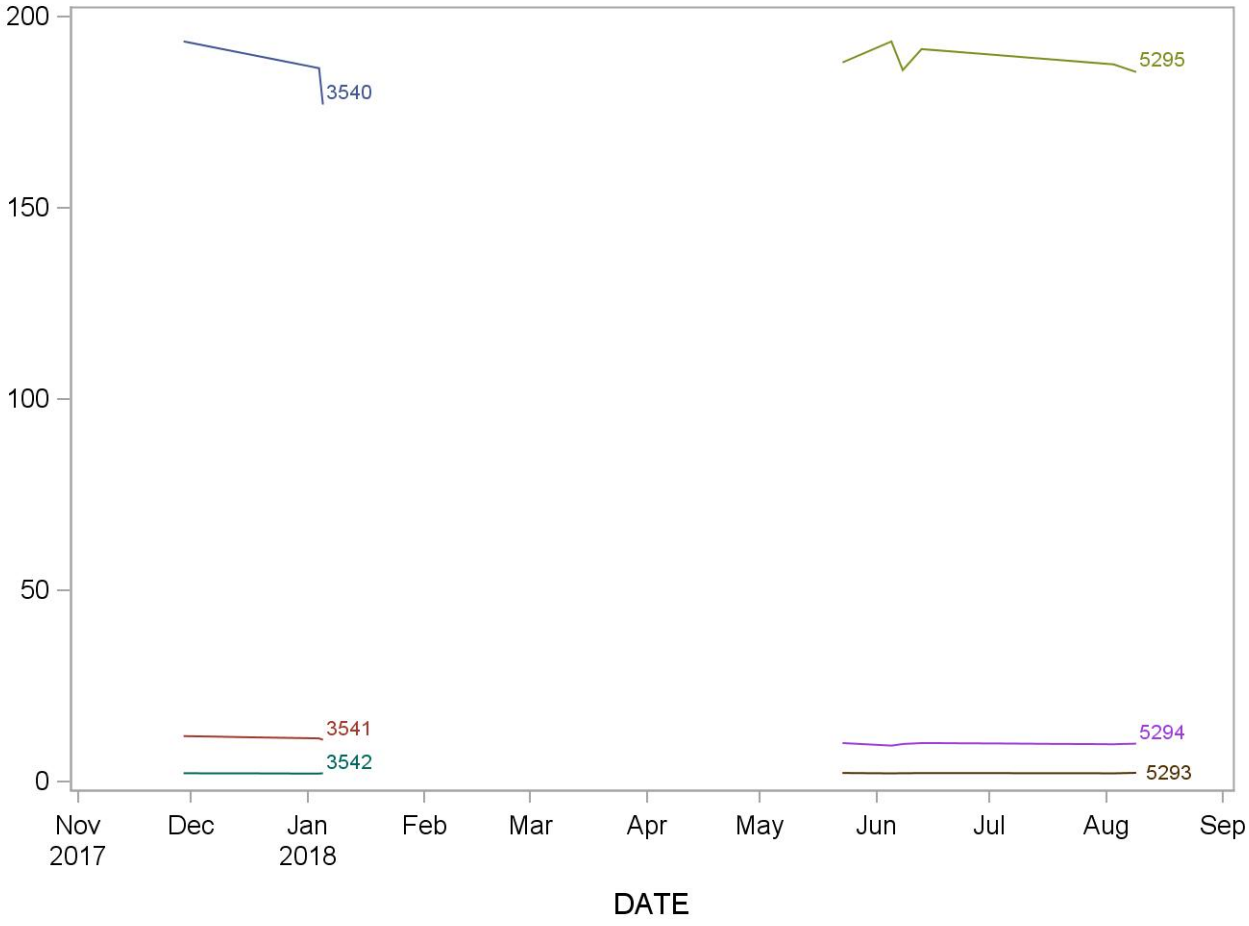
2013-2014 Summary Statistics and QC Chart for Genistein, Urine 2nd Collection (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	275.500	5.000	1.8
3541	3	29NOV17	05JAN18	43.083	1.032	2.4
3542	3	29NOV17	05JAN18	14.650	0.950	6.5
5293	6	23MAY18	09AUG18	14.333	0.894	6.2
5294	6	23MAY18	09AUG18	69.592	2.579	3.7
5295	6	23MAY18	09AUG18	382.583	10.111	2.6



2013-2014 Summary Statistics and QC Chart for o-Desmethylangolensin, Urine 2nd Collect (ng/mL)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3540	3	29NOV17	05JAN18	185.667	8.282	4.5
3541	3	29NOV17	05JAN18	11.383	0.480	4.2
3542	3	29NOV17	05JAN18	2.173	0.059	2.7
5293	6	23MAY18	09AUG18	2.227	0.049	2.2
5294	6	23MAY18	09AUG18	9.868	0.246	2.5
5295	6	23MAY18	09AUG18	188.667	3.173	1.7



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Appendix A: Method Performance Documentation

A. Accuracy

(1) Equol

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Analyte: **Equol**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration						Measured concentration				
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%) / SD (%)
Sample	1	0	3.40	3.59			Sample	1	9.34	10.00			
	2		3.77	3.87	3.76			2	9.45	9.91	9.68		99.7 / 2.0
	3		4.19	3.73				3	9.67	9.68			
Sample + Spike 1	1	4	7.38	7.81			Sample + Spike 1	1	16.6	17.2			
(Standard 3)	2		7.67	8.03	7.73	99.2	(Standard 5)	2	18.2	17.9	17.5	97.8	
	3		7.68	7.79				3	17.5	17.6			
Sample + Spike 2	1	6	10.1	9.94			Sample + Spike 2	1	23.4	23.0			
(Standard 4)	2		9.69	9.84	9.77	100.2	(Standard 6)	2	23.3	23.1	23.3	97.2	
	3		9.45	9.60				3	22.9	24.0			
Sample + Spike 3	1	8	11.9	11.9			Sample + Spike 3	1	27.2	29.6			
(Standard 5)	2		11.4	11.7	11.8	100.9	(Standard 7)	2	28.0	28.7	28.2	103	
	3		12.0	12.1				3	28.2	27.2			

(2) Daidzein

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Analyte: **Daidzein**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration						Measured concentration				
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%) / SD (%)
Sample	1	0	14.9	15.7			Sample	1	115	120			
	2		15.6	15.6	15.5			2	114	119	117		97.6 / 4.1
	3		15.1	16.0				3	115	118			
Sample + Spike 1	1	10	24.9	25.8			Sample + Spike 1	1	174	181			
(Standard 2)	2		25.0	25.4	25.1	95.8	(Standard 5)	2	187	193	183	94.3	
	3		24.3	25.0				3	177	185			
Sample + Spike 2	1	40	53.7	53.5			Sample + Spike 2	1	206	222			
(Standard 3)	2		52.0	55.4	53.6	95.2	(Standard 6)	2	221	221	219	102	
	3		52.9	53.8				3	218	225			
Sample + Spike 3	1	55	66.0	68.2			Sample + Spike 3	1	248	255			
(Standard 4)	2		65.7	69.8	67.7	94.9	(Standard 7)	2	241	242	247	104	
	3		67.3	69.0				3	242	251			

(3) O-DMA

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL

Analyte: **O-DMA**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration						Measured concentration				
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%) / SD (%)
Sample	1	0	2.09	2.02			Sample 1	0	8.90	8.72			93.7 / 2.1
	2		2.04	2.3	2.12		2		8.32	9.05	8.84		
	3		2.15	2.13			3		9.14	8.88			
Sample + Spike 1 (Standard 3)	1	3	4.94	5.22			Sample + Spike 1 (Standard 5) 1	5	13.4	13.9			
	2		4.88	4.78	4.94	93.9	2		13.4	13.5	13.5	94.0	
	3		4.95	4.87			3		13.4	13.6			
Sample + Spike 2 (Standard 4)	1	4	5.65	5.72			Sample + Spike 2 (Standard 6) 1	14	22.3	23.3			
	2		5.91	6.04	5.90	94.5	2		22.2	23.0	22.3	96.3	
	3		5.78	6.32			3		20.6	22.5			
Sample + Spike 3 (Standard 5)	1	5	6.87	6.85			Sample + Spike 3 (Standard 7) 1	20	26.8	26.9			
	2		6.54	6.73	6.79	93.4	2		26.3	27.1	26.8	89.8	
	3		6.77	6.98			3		26.7	27.0			

(4) Genistein

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL

Analyte: **Genistein**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration						Measured concentration				
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Mean recovery (%) / SD (%)
Sample	1	0	14.0	13.2			Sample 1	0	68.1	66.6			98.1 / 2.2
	2		13.2	12.4	13.3		2		58.8	65.5	63.3		
	3		13.0	13.8			3		59.1	61.8			
Sample + Spike 1 (Standard 3)	1	15	28.0	29.2			Sample + Spike 1 (Standard 5) 1	27	89.0	93.8			
	2		27.2	27.3	28.0	97.9	2		91.7	92.5	90.6	101.2	
	3		28.6	27.4			3		84.5	92.3			
Sample + Spike 2 (Standard 4)	1	22	35.0	37.8			Sample + Spike 2 (Standard 6) 1	55	110	121			
	2		32.2	33.9	35.1	99.0	2		119	118	115	94.6	
	3		35.1	36.3			3		108	116			
Sample + Spike 3 (Standard 5)	1	27	39.9	39.1			Sample + Spike 3 (Standard 7) 1	75	130	145			
	2		41.9	41.5	39.8	98.4	2		131	144	136	97.4	
	3		36.9	39.7			3		136	132			

(5) Enterolactone

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Analyte: **Enterolactone**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration					Measured concentration				Mean	SD
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	recovery (%)	(%)
Sample	1	0	33.8	34.6	34.3		Sample 1	0	436	457	439		101.3	2.6
	2		34.1	34.3			2		435	441				
	3		34.1	34.7			3		433	431				
Sample + Spike 1	1	10	44.4	45	44.8	104.8	Sample + Spike 1	315	734	766	749	98.6		
(Standard 2)	2		44	45.8			(Standard 5) 2		732	746				
	3		44.8	44.5			3		735	783				
Sample + Spike 2	1	150	188	196	190	104.0	Sample + Spike 2	550	986	1010	986	99.4		
(Standard 3)	2		185	191			(Standard 6) 2		977	979				
	3		189	193			3		977	985				
Sample + Spike 3	1	250	292	288	288	101.4	Sample + Spike 3	750	1200	1170	1185	99.5		
(Standard 4)	2		284	290			(Standard 7) 2		1210	1200				
	3		285	287			3		1160	1170				

(6) Enterodiol

Accuracy using Spike Recovery - fill in yellow shaded cells
Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%
Day 1 Run: 08/11/2017 PS
Day 2 Run: 08/17/2017 PS

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Analyte: **Enterodiol**

		Sample 1 QC Low (LU17440)					Sample 2 QC Med (MU17441)						
			Measured concentration					Measured concentration				Mean	SD
	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	Replicate	Spike concentration	Day 1	Day 2	Mean	Recovery (%)	recovery (%)	(%)
Sample	1	0	9.88	10.6	10.0		Sample 1	0	44.5	45.2	45.7		96.6	2.8
	2		9.98	9.94			2		43.9	47.9				
	3		9.96	9.58			3		46.7	46.2				
Sample + Spike 1	1	25	34.9	34.9	35.0	99.8	Sample + Spike 1	34	78.6	80.7	78.8	97.4		
(Standard 3)	2		34.6	35.1			(Standard 5) 2		75.3	77.8				
	3		36	34.2			3		80	80.6				
Sample + Spike 2	1	30	38.3	39.1	38.6	95.3	Sample + Spike 2	55	95.7	96.7	97.1	93.4		
(Standard 4)	2		38.1	37.7			(Standard 6) 2		98.6	98.5				
	3		38.3	39.9			3		95.3	97.7				
Sample + Spike 3	1	34	42.9	42.1	42.1	94.3	Sample + Spike 3	75	120	117	121	99.7		
(Standard 5)	2		41.6	42.4			(Standard 7) 2		124	123				
	3		43.2	40.2			3		118	121				

B. Precision

(1) Equol

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Data from QC Characterization files. The run dates are seen below.

All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **Equol**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	12.0	11.9	11.95	0.00	0.00	285.61
2 - 4/23/14	11.6	12.1	11.85	0.06	0.06	280.85
3 - 4/28/14	12.0	12.6	12.30	0.09	0.09	302.58
4 - 4/29/14	11.7	11.5	11.60	0.01	0.01	269.12
5 - 4/30/14	12.0	11.5	11.75	0.06	0.06	276.13
6 - 5/1/14	11.8	12.3	12.05	0.06	0.06	290.41
7 - 5/5/14	11.4	11.1	11.25	0.02	0.02	253.13
8 - 5/6/14	11.8	11.8	11.80	0.00	0.00	278.48
9 - 5/8/14	12.0	12.4	12.20	0.04	0.04	297.68
10 - 5/9/14	11.6	11.7	11.65	0.00	0.00	271.45
Grand sum	236.8	Grand mean	11.84			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.71	0.07	0.27	2.25
Between Run	1.70	0.19	0.24	2.05
Total	2.41		0.36	3.04

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	33.9	34.2	34.05	0.02	0.02	2318.81
2 - 4/23/14	33.5	34.6	34.05	0.30	0.30	2318.81
3 - 4/28/14	34.7	34.1	34.40	0.09	0.09	2366.72
4 - 4/29/14	32.2	32.8	32.50	0.09	0.09	2112.50
5 - 4/30/14	34.3	35.1	34.70	0.16	0.16	2408.18
6 - 5/1/14	32.1	34.4	33.25	1.32	1.32	2211.13
7 - 5/5/14	32.4	32.3	32.35	0.00	0.00	2093.05
8 - 5/6/14	33.6	33.8	33.70	0.01	0.01	2271.38
9 - 5/8/14	33.9	34.0	33.95	0.00	0.00	2305.21
10 - 5/9/14	34.7	34.5	34.60	0.01	0.01	2394.32
Grand sum	675.1	Grand mean	33.755			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	4.03	0.40	0.63	1.88
Between Run	12.08	1.34	0.69	2.03
Total	16.11		0.93	2.77

(2) Daidzein

Precision - fill in yellow shaded cells

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name:	Phytoestrogens
Method #:	4069
Matrix:	Urine
Units:	ng/mL

Data from QC Characterization files. The run dates are seen below.

All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **Daidzein**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	70.2	69.5	69.85	0.12	0.12	9758.05
2 - 4/23/14	73.2	72.4	72.80	0.16	0.16	10599.68
3 - 4/28/14	70.7	71.0	70.85	0.02	0.02	10039.45
4 - 4/29/14	67.4	69.8	68.60	1.44	1.44	9411.92
5 - 4/30/14	71.2	74.6	72.90	2.89	2.89	10628.82
6 - 5/1/14	67.3	71.8	69.55	5.06	5.06	9674.41
7 - 5/5/14	70.2	69.1	69.65	0.30	0.30	9702.25
8 - 5/6/14	71.1	69.0	70.05	1.10	1.10	9814.01
9 - 5/8/14	70.5	74.8	72.65	4.62	4.62	10556.05
10 - 5/9/14	64.9	68.5	66.70	3.24	3.24	8897.78
Grand sum	1407.2	Grand mean	70.36			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	37.93	3.79	1.95	2.77
Between Run	71.80	7.98	1.45	2.06
Total	109.73		2.43	3.45

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	584	590	587.00	9.00	9.00	689138.00
2 - 4/23/14	547	593	570.00	529.00	529.00	649800.00
3 - 4/28/14	574	580	577.00	9.00	9.00	665858.00
4 - 4/29/14	556	575	565.50	90.25	90.25	639580.50
5 - 4/30/14	582	586	584.00	4.00	4.00	682112.00
6 - 5/1/14	562	610	586.00	576.00	576.00	686792.00
7 - 5/5/14	565	583	574.00	81.00	81.00	658952.00
8 - 5/6/14	563	572	567.50	20.25	20.25	644112.50
9 - 5/8/14	566	575	570.50	20.25	20.25	650940.50
10 - 5/9/14	596	576	586.00	100.00	100.00	686792.00
Grand sum	11535	Grand mean	576.75			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2878	287.8	17.0	2.9
Between Run	1266	140.7	0.0	0.0
Total	4144		17.0	2.9

(3) O-DMA

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL
Data from QC Characterization files. The run dates are seen below.
All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **O-DMA**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	12.6	12.4	12.50	0.01	0.01	312.50
2 - 4/23/14	12.7	12.5	12.60	0.01	0.01	317.52
3 - 4/28/14	12.3	12.7	12.50	0.04	0.04	312.50
4 - 4/29/14	11.6	11.6	11.60	0.00	0.00	269.12
5 - 4/30/14	12.4	12.9	12.65	0.06	0.06	320.05
6 - 5/1/14	12.5	12.3	12.40	0.01	0.01	307.52
7 - 5/5/14	11.4	12.2	11.80	0.16	0.16	278.48
8 - 5/6/14	12.2	12.3	12.25	0.00	0.00	300.13
9 - 5/8/14	12.4	13.1	12.75	0.12	0.12	325.13
10 - 5/9/14	12.1	11.6	11.85	0.06	0.06	280.85
Grand sum	245.8	Grand mean	12.29			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	0.96	0.10	0.31	2.52
Between Run	2.90	0.32	0.34	2.74
Total	3.86		0.46	3.72

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	189	191	190.00	1.00	1.00	72200.00
2 - 4/23/14	180	198	189.00	81.00	81.00	71442.00
3 - 4/28/14	196	199	197.50	2.25	2.25	78012.50
4 - 4/29/14	185	187	186.00	1.00	1.00	69192.00
5 - 4/30/14	199	201	200.00	1.00	1.00	80000.00
6 - 5/1/14	187	205	196.00	81.00	81.00	76832.00
7 - 5/5/14	179	187	183.00	16.00	16.00	66978.00
8 - 5/6/14	189	192	190.50	2.25	2.25	72580.50
9 - 5/8/14	182	195	188.50	42.25	42.25	71064.50
10 - 5/9/14	229	231	230.00	1.00	1.00	105800.00
Grand sum	3901	Grand mean	195.05			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	457.50	45.75	6.76	3.47
Between Run	3211.45	356.83	12.47	6.39
Total	3668.95		14.19	7.27

(4) Genistein

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL
Data from QC Characterization files. The run dates are seen below.
All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **Genistein**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	44.9	47.0	45.95	1.10	1.10	4222.81
2 - 4/23/14	46.0	48.3	47.15	1.32	1.32	4446.25
3 - 4/28/14	44.1	46.0	45.05	0.90	0.90	4059.01
4 - 4/29/14	43.4	45.7	44.55	1.32	1.32	3969.41
5 - 4/30/14	46.2	47.1	46.65	0.20	0.20	4352.45
6 - 5/1/14	45.7	45.9	45.80	0.01	0.01	4195.28
7 - 5/5/14	40.6	40.7	40.65	0.00	0.00	3304.85
8 - 5/6/14	44.7	45.8	45.25	0.30	0.30	4095.13
9 - 5/8/14	47.5	45.9	46.70	0.64	0.64	4361.78
10 - 5/9/14	46.0	45.8	45.90	0.01	0.01	4213.62
Grand sum	907.3	Grand mean	45.365			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	11.64	1.16	1.08	2.38
Between Run	60.89	6.77	1.67	3.69
Total	72.53		1.99	4.39

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	299	287	293.00	36	36	171698
2 - 4/23/14	273	311	292.00	361	361	170528
3 - 4/28/14	304	280	292.00	144	144	170528
4 - 4/29/14	271	276	273.50	6.25	6.25	149604.5
5 - 4/30/14	281	308	294.50	182.25	182.25	173460.5
6 - 5/1/14	267	289	278.00	121	121	154568
7 - 5/5/14	278	273	275.50	6.25	6.25	151800.5
8 - 5/6/14	273	299	286.00	169	169	163592
9 - 5/8/14	307	292	299.50	56.25	56.25	179400.5
10 - 5/9/14	312	333	322.50	110.25	110.25	208012.5
Grand sum	5813	Grand mean	290.65			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	2384.50	238.45	15.44	5.31
Between Run	3644.05	404.89	9.12	3.14
Total	6028.55		17.94	6.17

(5) Enterolactone

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL
Data from QC Characterization files. The run dates are seen below.
All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **Enterolactone**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	351	348	349.50	2.25	2.25	244300.5
2 - 4/23/14	327	341	334.00	49	49	223112
3 - 4/28/14	325	343	334.00	81	81	223112
4 - 4/29/14	326	330	328.00	4	4	215168
5 - 4/30/14	327	331	329.00	4	4	216482
6 - 5/1/14	329	337	333.00	16	16	221778
7 - 5/5/14	328	320	324.00	16	16	209952
8 - 5/6/14	334	322	328.00	36	36	215168
9 - 5/8/14	335	352	343.50	72.25	72.25	235984.5
10 - 5/9/14	328	320	324.00	16	16	209952
Grand sum	6654	Grand mean	332.7			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	593.00	59.30	7.70	2.31
Between Run	1223.20	135.91	6.19	1.86
Total	1816.20		9.88	**2.97**

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	1230	1160	1195.00	1225	1225	2856050
2 - 4/23/14	1110	1270	1190.00	6400	6400	2832200
3 - 4/28/14	1190	1180	1185.00	25	25	2808450
4 - 4/29/14	1110	1130	1120.00	100	100	2508800
5 - 4/30/14	1240	1240	1240.00	0	0	3075200
6 - 5/1/14	1120	1200	1160.00	1600	1600	2691200
7 - 5/5/14	1170	1130	1150.00	400	400	2645000
8 - 5/6/14	1150	1110	1130.00	400	400	2553800
9 - 5/8/14	1220	1200	1210.00	100	100	2928200
10 - 5/9/14	1230	1210	1220.00	100	100	2976800
Grand sum	23600	Grand mean	1180			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	20700.00	2070.00	45.50	3.86
Between Run	27700.00	3077.78	22.45	1.90
Total	48400.00		50.73	**4.30**

(6) Enterodiol

Precision - fill in yellow shaded cells
Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: Phytoestrogens
Method #: 4069
Matrix: Urine
Units: ng/mL
Data from QC Characterization files. The run dates are seen below.
All Analytes for Med and High QC are ≤ 15% for the total relative standard deviation

Analyte: **Enterodiol**

Med QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	39.7	40.0	39.85	0.02	0.02	3176.05
2 - 4/23/14	40.0	41.6	40.80	0.64	0.64	3329.28
3 - 4/28/14	40.4	41.0	40.70	0.09	0.09	3312.98
4 - 4/29/14	39.1	39.8	39.45	0.12	0.12	3112.61
5 - 4/30/14	40.3	40.9	40.60	0.09	0.09	3296.72
6 - 5/1/14	37.2	40.2	38.70	2.25	2.25	2995.38
7 - 5/5/14	39.9	38.9	39.40	0.25	0.25	3104.72
8 - 5/6/14	39.9	39.7	39.80	0.01	0.01	3168.08
9 - 5/8/14	41.1	43.5	42.30	1.44	1.44	3578.58
10 - 5/9/14	40.4	39.9	40.15	0.06	0.06	3224.05
Grand sum	803.5	Grand mean	40.175			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	9.96	1.00	1.00	2.48
Between Run	17.82	1.98	0.70	1.75
Total	27.78		1.22	**3.04**

High QC

Run	Result 1	Result 2	Mean	SS 1	SS 2	2*mean^2
1 - 4/22/14	194	178	186.00	64.00	64.00	69192.00
2 - 4/23/14	180	193	186.50	42.25	42.25	69564.50
3 - 4/28/14	191	187	189.00	4.00	4.00	71442.00
4 - 4/29/14	184	187	185.50	2.25	2.25	68820.50
5 - 4/30/14	194	192	193.00	1.00	1.00	74498.00
6 - 5/1/14	195	182	188.50	42.25	42.25	71064.50
7 - 5/5/14	190	186	188.00	4.00	4.00	70688.00
8 - 5/6/14	188	192	190.00	4.00	4.00	72200.00
9 - 5/8/14	189	194	191.50	6.25	6.25	73344.50
10 - 5/9/14	207	208	207.50	0.25	0.25	86112.50
Grand sum	3811	Grand mean	190.55			

	Sum squares	Mean Sq Error	Std Dev	Rel Std Dev (%)
Within Run	340.50	34.05	5.84	3.06
Between Run	740.45	82.27	4.91	2.58
Total	1080.95		7.63	**4.00**

C. Stability

(1) Equol

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Quality material 1 = MU17441		**Quality material 2 = HU17442**	
Analyte:	**Equol**		

Quality material 1					**Quality material 2**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	11.2	10.8	10.8	11.3	Replicate 1	67.4	68.5	70.7	70.8
Replicate 2	10.9	11.0	10.9	11.1	Replicate 2	70.6	68.4	68.8	69.1
Replicate 3	11.4	10.9	10.8	10.9	Replicate 3	67.4	71.1	68.2	69.8
Mean	11.2	10.9	10.8	11.1	Mean	68.5	69.3	69.2	69.9
% difference from initial measurement	--	**-2.4**	**-3.0**	**-0.6**	**% difference from initial measurement**	--	**1.3**	**1.1**	**2.1**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens						
Method #:	4069						
Matrix:	Urine		Initial Measurment			Long-term stability		
Units:	ng/mL		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
		Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Quality material 1 = MU09441e				**Quality material 2 = HU09441e**				
Analyte:	**Equol**							

Quality material 1			**Quality material 2**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	12.5	11.2	Replicate 1	36.2	33.7
Replicate 2	12.0	12.4	Replicate 2	35.8	33.5
Replicate 3	12.3	11.7	Replicate 3	34.9	34.0
Mean	12.3	11.8	Mean	35.6	33.7
% difference from initial measurement	--	**-4.1**	**% difference from initial measurement**	--	**-5.3**

(2) Daidzein

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Analyte:	**Daidzein**		

Quality material 1					**Quality material 2**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	135	136	137	136	Replicate 1	1100	1080	1080	1100
Replicate 2	136	136	136	135	Replicate 2	1080	1070	1080	1080
Replicate 3	139	135	135	131	Replicate 3	1030	1130	1070	1090
Mean	137	136	136	134	Mean	1070	1093	1077	1090
% difference from initial measurement	--	**-0.7**	**-0.5**	**-2.0**	**% difference from initial measurement**	--	**2.2**	**0.6**	**1.9**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

			Initial Measurment			Long-term stability		
Method name:	Phytoestrogens		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Method #:	4069	Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Matrix:	Urine							
Units:	ng/mL							
Analyte:	**Daidzein**							

Quality material 1			**Quality material 2**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	72.4	71.0	Replicate 1	607	576
Replicate 2	71.4	69.9	Replicate 2	582	578
Replicate 3	69.9	68.5	Replicate 3	595	585
Mean	71.2	69.8	Mean	595	580
% difference from initial measurement	--	**-2.0**	**% difference from initial measurement**	--	**-2.5**

(3) O-DMA

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15°C for 24 hours then stored at 5°C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Analyte:	**O-DMA**		

Quality material 1	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	10.7	10.3	10.4	9.8
Replicate 2	9.8	10.1	10.1	9.65
Replicate 3	10.0	10.4	9.8	10.6
Mean	10	10.3	10.1	10.0
% difference from initial measurement	--	**1.0**	**-0.7**	**-1.5**

Quality material 2	**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	193	193	195	188
Replicate 2	195	193	195	189
Replicate 3	195	190	193	196
Mean	194	192	194	191
% difference from initial measurement	--	**-1.2**	**0.0**	**-1.7**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

			Initial Measurment			Long-term stability		
Method name:	Phytoestrogens		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Method #:	4069	Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Matrix:	Urine							
Units:	ng/mL							
Analyte:	**O-DMA**							

Quality material 1	**Initial measurement**	**Long-term stability**
Replicate 1	12.6	11.4
Replicate 2	11.6	12.1
Replicate 3	12.2	11.7
Mean	12.1	11.7
% difference from initial measurement	--	**-3.3**

Quality material 2	**Initial measurement**	**Long-term stability**
Replicate 1	204	188
Replicate 2	203	193
Replicate 3	198	201
Mean	202	194
% difference from initial measurement	--	**-3.8**

(4) Genistein

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Analyte:	**Genistein**		

Quality material 1					**Quality material 2**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	84.5	77.1	73.5	72.1	Replicate 1	408	399	379	392
Replicate 2	72.5	67.9	73.5	72.2	Replicate 2	398	421	414	394
Replicate 3	74.9	71.5	75.0	73.6	Replicate 3	424	403	391	363
Mean	77.3	72.2	74.0	72.6	Mean	410	408	395	383
% difference from initial measurement	--	**-6.6**	**-4.3**	**-6.0**	**% difference from initial measurement**	--	**-0.6**	**-3.7**	**-6.6**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

			Initial Measurment			Long-term stability		
Method name:	Phytoestrogens							
Method #:	4069							
Matrix:	Urine		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Units:	ng/mL	Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Analyte:	**Genistein**							

Quality material 1			**Quality material 2**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	48.7	46.6	Replicate 1	306	291
Replicate 2	51.0	44.6	Replicate 2	289	310
Replicate 3	45.5	43.7	Replicate 3	303	302
Mean	48.4	45.0	Mean	299	301
% difference from initial measurement	--	**-7.1**	**% difference from initial measurement**	--	**0.6**

(5) Enterolactone

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Analyte:	**Enterolactone**		

Quality material 1					**Quality material 2**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	476	468	477	488	Replicate 1	1160	1180	1190	1160
Replicate 2	486	474	495	476	Replicate 2	1170	1220	1180	1170
Replicate 3	465	483	480	489	Replicate 3	1180	1170	1180	1180
Mean	475.6666667	475	484	484	Mean	1170	1190	1183	1170
% difference from initial measurement	--	**-0.1**	**1.8**	**1.8**	**% difference from initial measurement**	--	**1.7**	**1.1**	**0.0**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

			Initial Measurment			Long-term stability		
Method name:	Phytoestrogens		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Method #:	4069	Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Matrix:	Urine							
Units:	ng/mL							
Analyte:	**Enterolactone**							

Quality material 1			**Quality material 2**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	340	331	Replicate 1	1290	1200
Replicate 2	338	327	Replicate 2	1220	1200
Replicate 3	335	329	Replicate 3	1200	1240
Mean	338	329	Mean	1237	1213
% difference from initial measurement	--	**-2.6**	**% difference from initial measurement**	--	**-1.9**

(6) Enterodiol

Stability - fill in yellow shaded cells

Freeze and thaw stability = Assess for a minimum of 3 freeze-thaw cycles; conditions should mimic intended sample handling conditions
Describe condition: three times frozen at -80°C and then thawed (3 freeze-thaw cycles)
Bench-top stability = Assess short-term stability for length of time needed to handle study samples (typically at room temperature)
Describe condition: original samples (not yet prepared for instrument analysis) stored at room temperature for 8 hours
Processed sample stability = Assess short-term stability of processed samples, including resident time in autosampler
Describe condition: processed samples (ready for instrument analysis) stored at 15˚C for 24 hours then stored at 5˚C for 2 months

All stability sample results should be within ±15% of nominal concentration

Method name:	Phytoestrogens	**Data from: 10/6/2017**	**Processed sample stability from: 12/06/2017**
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		
Analyte:	**Enterodiol**		

Quality material 1					**Quality material 2**				
	Initial measurement	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**		**Initial measurement**	**Three freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**
Replicate 1	49.4	51.7	51.9	51.8	Replicate 1	162	162	162	169
Replicate 2	52.4	51.3	50.5	51.6	Replicate 2	159	163	159	169
Replicate 3	50.9	49.4	50.5	53.3	Replicate 3	159	159	159	162
Mean	50.9	50.8	51.0	52.2	Mean	160	161	160	167
% difference from initial measurement	--	**-0.2**	**0.1**	**2.6**	**% difference from initial measurement**	--	**0.8**	**0.0**	**4.2**

Long-term stability = Assess long-term stability that equals or exceeds time between date of first sample collection and date of last sample analysis
Describe condition: example: samples stored at -80°C for 2 years

All stability sample results should be within ±15% of nominal concentration

			Initial Measurment			Long-term stability		
Method name:	Phytoestrogens		Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Method #:	4069	Run Date	8/20/2015	8/21/2015	8/25/2015	8/29/2017	9/8/2017	9/22/2017
Matrix:	Urine							
Units:	ng/mL							
Analyte:	**Enterodiol**							

Quality material 1			**Quality material 2**		
	Initial measurement	**Long-term stability**		**Initial measurement**	**Long-term stability**
Replicate 1	44.8	37.4	Replicate 1	203	183
Replicate 2	40.9	39.7	Replicate 2	198	197
Replicate 3	44.2	38.3	Replicate 3	198	182
Mean	43.3	38.5	Mean	200	187
% difference from initial measurement	--	**-11.2**	**% difference from initial measurement**	--	**-6.2**

D. LOD, Specificity, and Fit for Intended Use

LOD, specificity and fit for intended use - fill in yellow shaded cells

Method name:	Phytoestrogens		
Method #:	4069		
Matrix:	Urine		
Units:	ng/mL		

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
Equol	0.1	yes	yes
Daidzein	0.4	yes	yes
O-DMA	0.1	yes	yes
Genistein	0.2	yes	yes
Enterolactone	0.2	yes	yes
Enterodiol	0.9	*yes	yes

*Known interference that is excluded from the peak integration

*10-20% Peak Interference found in 6% of human samples (n=73)

*2-6% Peak Interference found in 8% of human samples (n=73)

Appendix B: Ruggedness Testing*

A. Experiments

(1) Deconjugation of phytoestrogens

a) Principle: Phytoestrogens in urine occur in conjugated forms (e.g., glucuronides, sulfides). Our analysis method quantifies total phytoestrogens after a complete enzymatic deconjugation with Helix pomatia. Incomplete deconjugation would lead to erroneously low results.

b) Proposal: To vary the following conditions, which are specific to how the deconjugation process is conducted: enzyme concentration, incubation time, buffer pH, and buffer molarity.

(2) Sample Stability

a) Principle: Due to instrument problems or a number of other potential issues that may prevent immediate sample analysis, it is important to determine whether phytoestrogens in urine are stable after going through the sample preparation process. A decrease in stability over time could result in erroneously low results.

b) Proposal: Inject the same sample set across multiple days, all the while holding the samples in the HPLC autosampler at a constant 15 °C throughout.

* Ruggedness testing experiments shown here were conducted as part of the validation of the previous APPI-based method **[17]** and were not repeated for the purposes of validating the ESI-based method described in this document. The sample preparation process is identical for both methods and four of the five ruggedness testing experiments are specific to sample preparation, so the results will be the same. The fifth experiment relates to storage after preparation, which will also yield identical results between methods.

B. Findings

(1) Deconjugation of phytoestrogens

Factor	Method specifies	Results (ng/mL)	Lower level	Results (ng/mL)	Higher level	Results (ng/mL)
Enzyme concentration	120 units /sample	DAZ: 66.1 DMA: 12.6 EQU: 13.2 ETD: 43.1 ETL: 348 GNS: 46.6	60 units /sample	DAZ: 70.6 DMA: 12.5 EQU: 13.3 ETD: 41.7 ETL: 361 GNS: 47.1	240 units /sample	DAZ: 66.6 DMA: 12.8 EQU: 13.6 ETD: 43.1 ETL: 357 GNS: 47.4
Incubation time	12 hours	DAZ: 72.5 DMA: 13.5 EQU: 14.3 ETD: 44.5 ETL: 385 GNS: 44.9	4 hours	DAZ: 72.9 DMA: 12.8 EQU: 14.1 ETD: 39.9 ETL: 383 GNS: 42.7	24 hours	DAZ: 75.9 DMA: 13.3 EQU: 14.2 ETD: 49.1 ETL: 388 GNS: 45.4
Buffer pH	5.0	DAZ: 72.4 DMA: 12.4 EQU: 13.9 ETD: 47.1 ETL: 385 GNS: 46.2	4.5	DAZ: 70.3 DMA: 11.9 EQU: 13.2 ETD: 44.2 ETL: 384 GNS: 42.9	5.5	DAZ: 73.4 DMA: 12.8 EQU: 13.5 ETD: 45.9 ETL: 392 GNS: 49.5
Buffer molarity[†]	2.5 M	DAZ: 70.6 DMA: 13.3 EQU: 13.6 ETD: 43.5 ETL: 381 GNS: 44.2	0.25 M	DAZ: 71.9 DMA: 13.9 EQU: 13.6 ETD: 43.9 ETL: 373 GNS: 44.2	1.25 M	DAZ: 71.6 DMA: 13.7 EQU: 13.4 ETD: 44.9 ETL: 387 GNS: 44.8

Enzyme concentration: Varying the enzyme concentration over the specified range does have implications on assay accuracy. Given the current incubation time of 12 hours, a minimum of 120 units of enzyme must be used per sample in order to achieve complete analyte deconjugation. This issue is most apparent in samples near the upper end of the calibration range, and is typically only seen with enterodiol.

Incubation time: Varying the incubation time over the specified range does have implications on assay accuracy. It has been shown that sample incubation times of less than 4 hours can lead to inaccurate (low) concentrations as a result of the deconjugation process not being carried to completion. Slight, but noticeable, increases in concentration have also been shown at incubation time points between 4-6 hours. To compensate for these findings, a conservative minimum time of 12 hours must be utilized for this method.

[†] Test conditions are one-sided tests only (both higher and lower conditions were not experimentally possible).

Buffer pH: Varying the buffer pH over the specified range has no effect on analysis results.

Buffer molarity: According to the data presented here, the molarity of the buffer used appears to have no negative impact across the specified range. However, other studies have indicated that the pH of some urine samples is sufficiently high enough to cause analyte deprotonation, resulting in poor chromatographic performance. Therefore, it is necessary to use no less than the method specified volume of 2.5 M buffer in order to maintain chromatographic separation and prevent sensitivity loss.

(2) Sample Stability

Factor	Method specifies	Results (ng/mL)	Medium level	Results (ng/mL)	Highest level	Results (ng/mL)
After-prep stability	same day	DAZ: 72.0 DMA: 13.4 EQU: 13.4 ETD: 42.6 ETL: 381 GNS: 40.6	3 days	DAZ: 75.7 DMA: 14.1 EQU: 13.8 ETD: 43.4 ETL: 389 GNS: 42.5	7 days	DAZ: 73.0 DMA: 14.1 EQU: 13.7 ETD: 43.3 ETL: 385 GNS: 44.2

After-prep stability: Degradation of phytoestrogen analytes is not noticed over the specified range.

Appendix C: Preparation of mixed working standard solutions for analytes and internal standards‡

Analyte	Analytical Standards										Int. Std.
	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9	^{13}C
Equol	0.1	0.3	1	4	6	8	14	18	60	100	9.6
Daidzein	0.4	1	10	40	55	70	100	125	1000	1600	7
O-DMA	0.1	0.4	1	3	4	5	14	20	110	300	1
Genistein	0.2	0.4	2	15	22	27	55	75	325	730	5
Enterolactone	0.2	2	10	150	250	315	550	750	2100	3300	6.8
Enterodiol	0.09	0.3	2	25	30	34	55	75	175	320	5.4

‡ Table specifies amount (ng) in 100 µL spike of Mixed Working Analytical Standard Solutions and 50 uL spike of 13C-labeled Mixed Working Internal Standard Solutions (after 5x dilution per method specifications)

Appendix D: Target values for quality control specimens

	Concentration (ng/mL)		
Analyte	**Low**	**Medium**	**High**
Equol	5	12	35
Daidzein	20	70	582
O-DMA	2	12	197
Genistein	15	45	291
Enterolactone	58	332	1246
Enterodiol	17	40	188

Appendix E: Urine phytoestrogen levels from the U.S. population[§]

	Urine concentration (μg/L)				
Analyte	**Geometric mean**	**5th**	**50th**	**95th**	**Sample Size**
Daidzein	66.6	5.35	60.4	1,170	5,122
Enterodiol	38.6	2.48	43.9	377	5,122
Enterolactone	290	10.9	390	2,740	5,122
Equol	8.21	0.953	8.33	64.8	5,117
Genistein	29.9	2.45	26.1	523	5,122
O-DMA	4.80	<LOD	4.09	251	5,109

[§] Representative sample of the U.S. population aged 6 years and older from the National Health and Nutrition Examination Survey, 2003-2006, as published in the National Report on Biochemical Indicators of Diet and Nutrition in the U.S. Population (CDC – 2012)

Appendix F: 96-well plate layout for sample preparation**

STD 0	STD 8	QC M	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 1	STD 9	QC H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 2	Double Blank	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 3	Blank	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 4	QC L	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 5	QC M	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 6	QC H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
STD 7	QC L	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

** Suggested format for manual sample preparation. Legend: STD (standard); QC (quality control); UNK (unknown).

Appendix G: Solvent gradient for phytoestrogens LC-ESI-MS/MS sample acquisition method

	Gradient composition (%)	
Time (min)	**Water (A)**	**Methanol (B)**
0	65	35
0.5	65	35
2.5	5	95
4.5	5	95
5	65	35
7	65	35

Appendix H: Mass spectrometric parameters

The values below are of mass spectrometric parameters based on previous acquisition methods for phytoestrogens. These values are only provided as a close approximation; actual values should be determined by optimizing and calibrating the instrument as suggested in the instrument manual.

Analyte	MRM Transitions (*m/z*)			Dwell Time (ms)	Mass Spectrometer Potentials (V)* - API 6500		
	Type	Mol. Ion	Prod. Ion		DP	CE	CXP
Equol	Quant.	241	121	20	-30	-19	-14
	Conf.	241	93	20	-30	-35	-12
	IS	244	122	20	-30	-21	-10
Daidzein	Quant.	253	208	20	-50	-40	-12
	Conf.	253	132	20	-50	-49	-14
	IS	256	226	20	-50	-45	-13
O-Desmethylangolensin	Quant.	257	136	20	-50	-30	-15
	Conf.	257	108	20	-50	-36	-12
	IS	260	137	20	-50	-32	-10
Genistein	Quant.	269	133	20	-50	-41	-10
	Conf.	269	224	20	-50	-36	-10
	IS	272	63	20	-50	-65	-10
Enterolactone	Quant.	297	107	20	-50	-35	-11
	Conf.	297	121	20	-50	-32	-12
	IS	300	108	20	-50	-35	-11
Enterodiol	Quant.	301	106	20	-50	-45	-11
	Conf.	301	253	20	-50	-32	-11
	IS	304	256	20	-50	-33	-10
Umbelliferone	IS	175	133	20	-50	-30	-15
$^{13}C_4$-Umbelliferone	IS	179	106	20	-50	-33	-10

*Entrance potential (EP) was held contanst at -10V for all transitions